INHIBITION OF HUMAN COMPLEMENT BY EXTRACELLULAR LIPOTEICHOIC ACID FROM *STREPTOCOCCUS MUTANS* BHT

By

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A DISSERTATION PRESENTED TO THE
GRADUATE COUNCIL OF THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA
1977

ACKNOWLEDGEMENTS

In all sincerity, no occasion or project thus far undertaken has had a more humbling effect on my life than the completion of this Ph.D.dissertation. It is unfortunate that only now in retrospect can I clearly see the tremendous debt I owe for the help, patience, understanding, and knowledge so generously contributed by my mentor, my friends and wife.

If I were to formally thank everyone who contributed in someway to the successful completion of my Ph.D. dissertation, it is likely that my acknowledgements would read like the listings of a telephone book. I do however feel compelled to thank a few very special people.

First of all I would like to thank my mentor and friend, Dr. E.M. Hoffmann. Ed possesses the rare ability to earn respect rather than having to demand it. His tolerance for my idiosyncrasies was unlimited (almost). He gave me direction yet left me with alternatives; he challenged my intellect, yet never made me feel ignorant; he provided the foundation on which I am still building my scientific character. Most of all, he was (and is) a friend.

I would also like to thank the members of our laboratory "family" (Suzanne, Jean, Bert and Tom) for their help, patience and tolerance during these difficult last days. One cannot help but reflect upon the many events that shape the complex web of friendships within the laboratory. All of you will always be regarded as the closest of friends.

I wish to express my gratitude to Ron Craig not only for his friendship, but also for the professional technical assistance that he afforded.

I would also like to express my appreciation to the employees of Teaching Resources, for dedication above and beyond the call of duty. I would

especially like to acknowledge the professional artistic assistance of Margie Summers, Margie Niblack and John Knaub.

I would like to thank Steve Hurst for being there when I needed a friend and for helping with some of the last minute photography.

The typist, Joanne Hall, deserves a particularly special mention. If not for her personal concern and dedication the deadlines would never have been met. She worked on this dissertation under conditions for which no degree of monetary reinbursement could possibly compensate. I thank you Joanne and I sincerely hope you never have to go through that again!

Finally, and most importantly, I wish to thank my wife Lyn for her infinite patience and encouragement. Her attitudes, her ideals, her "being" is so much a part of me that it would be hopelessly futile to list all the things for which I am indebted to her. She is a friend and lover, a typist and an occasional laboratory technician. She is my driving force in life and rightfully so, I dedicate this dissertation to her.

" And with one loud worrawarrawarrawarrawarra he jumped at the end of the tablecloth, pulled it to the ground, wrapped himself up in it three times, rolled to the other end of the room, and after a terrible struggle got his head into daylight again and said cheerfully

-- have I won?"

(from "The House at Pooh Corner" by A.A. Milne)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GLOSSARY OF ABBREVIATIONS

A:	Antibody
C:	Complement
C1, C2---C9:[a]	Complement components. Horizontal bars above the component designation denotes a biologically active state.
CVF:	Cobra venom factor
E:	Erythrocyte
EDTA:	(Disodium) Ethylenediamine tetraacetic acid
EGTA:	Ethyleneglycol-bis (ß Amino Ethyl Ether) N,N^{1} tetra acetic acid
LTAcx:	Crude extracellular lipoteichoic acid
LTApcx:	Extracellular LTA purified via phosphatidyl-choline vesicle adsorbtion
LTAppx:	Partially purified extracellular LTA
LTAosx:	Extracellular LTA purified via Octyl Sepharose gel column adsorbtion
LPS:	Lipopolysaccharide
PHA:	Passive hemagglutination
PHAg:	Passive hemagglutination (modified method)
TA:	Teichoic acid
TAME:	p-Tosyl-l-arginine methylester

[a] All complement nomenclature follows the WHO recommendations (Bull. Wld. Hlth. Org. 39:939, 1968).

Abstract of Dissertation Presented to the
Graduate Council of the University of Florida
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy

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December, 1977

Chairman: Edward M. Hoffmann
Major Department: Microbiology and Cell Science

A number of biological and chemical similarities exist between the lipopolysaccharides (LPS) of gram negative microorganisms and the lipoteichoic acids (LTA) of gram positive organisms. The potent affects of LPS on the complement system are well documented; however, the effect of LTA on this host defense system has not been adequately studied. Furthermore, all studies thus far conducted have been limited to the interaction of LTA with whole fluid phase complement. In this investigation it was demonstrated that extracellular LTA from the cariogenic microorganism Streptococcus mutans BHT was not only capable of spontaneously binding to sheep erythrocyte target cells but was also capable of rendering them refractory to complement mediated lysis. Purification of the LTA to homogeneity was achieved by a combination of gel filtration and adsorbtion to phospholipid choline vesicles (artificial membranes). By utilizing various cellular complement component intermediate complexes and functionally purified complement components, experiments were conducted to define the site and mechanism of inhibition

by LTA. The site of inhibition was determined to occur between the formation of the SAC$\overline{1}$ and SAC$\overline{142}$ complex. Because C$\overline{1}$ is no longer necessary after formation of the C3 convertase (SAC$\overline{42}$), lack of inhibition after this step implies a direct effect on C$\overline{1}$ activity. Although experimental data derived from utilizing C$\overline{1}$, C1q, C1s, and C$\overline{1s}$ were suggestive, data did not unequivocally establish this as the precise mechanism of inhibition. No evidence for fluid phase consumption of hemolysin Ab, C$\overline{1}$, C4, or C2 by LTA could be demonstrated. Evidence for the inhibitory activity of LTA from several unrelated genera is presented and the possible role of LTA in periodontal disease is discussed.

INTRODUCTION

As reviewed by Wicken and Knox (1,2), a number of chemical and biological similarities exist between the lipopolysaccharides (LPS) of gram negative bacteria and the lipoteichoic acids (LTA) of gram positive organisms. Because of these similitudes our laboratory began to investigate whether LTA possessed anticomplementary activity analogous to that associated with the LPS endotoxin (3-8). Although there have been concentrated efforts to define the site and mechanism of LPS inhibition of complement, very few investigators have reported data on the possible effects of LTA on the complement system (9). This is somewhat surprising since the interaction of LTA, LPS, and complement almost certainly play a significant role in the etiology of periodontal diseases. Bacterial products and serum components in the gingival crevices of the oral cavity have been shown to activate complement by both the classical (10,11) and the alternative pathways (12,13). In fact recent evidence suggests that bone loss (a major clinical manifestation of acute periodontal disease) may occur via osteoclast activation due to the interaction of complement and prostaglandin E (14). Prostaglandins are naturally occurring cyclized derivatives of unsaturated long chain fatty acids (15) and their concentrations are dramatically elevated in inflamed gingival tissues (16). It is of interest to note that both LTA and LPS are also capable of initiating osteoclast mediated bone resorbtion (17) and this activity proceeds without the contribution of complement or prostaglandins. The potential for synergism cannot be overlooked, and indeed LPS endotoxins have long been implicated as participants in the development of periodontal lesions (4-8). Analogous LTA activity could be of significant clinical import especially

in light of the fact that gram positive bacteria represent the major cellular constituent of dental plaque at the early stages of plaque formation (18). Most of the gram positive organisms found in dental plaque have been isolated, cultured, and identified. The production of copious amounts of extracellular LTA by several of these organisms has been well established (19,20). In fact, growing under conditions estimated to reflect the growth rate in the oral cavity, Wicken and Knox have shown that the cariogenic bacterium Streptococcus mutans BHT produces some eleven fold greater amount of extracellular LTA in the culture fluid than that contained within the cells themselves (1,2). Therefore, if an effect on complement by LTA can be demonstrated in vitro, an in vivo model can be readily envisioned. Preliminary experimentation with a crude LTA containing extract from S. mutans BHT did indeed indicate that complement activity was consumed.[1] However, consumption or alteration of complement activity can be due to a number of specific or non-specific factors. Because of the complexity of this system, a thorough understanding of the possible interactions is necessary before any model attempting to define a site and mechanism of inhibition can be elucidated.

The complement system of vertebrates is comprised of at least eighteen discrete plasma proteins capable of interacting in a specific and sequential fashion. There are two pathways by which this biochemical cascade may be initiated and they are referred to as the classical and the alternative pathways of complement activation. However, regardless of how the activation scheme is initiated, the biological consequences of activation are the same for both pathways:

[1] Silvestri et al. 1976. Abst. Ann. Meeting, ASM, p77.

1). phlogogenic activity mediated via complement reaction by-products
2). increased opsonic susceptibility of foreign substances
3). irreversible physiochemical membrane damage--and ultimately, cytolysis--of susceptable target cells. Although the importance of complement as a component of the host defense system has been suspected for quite some time, only recently has its biomedical significance been firmly established. Indeed, the participation of complement in host resistance to infections and in several disease mechanisms is a topic which has generated considerable research interest in recent years (21,22).

The classical pathway of complement contains eleven discrete glycoproteins representing nine distinct components referred to sequentially as C1 through C9. C1 is actually a multimolecular complex of three distinct proteins (C1q, C1r, and C1s) and the aggregate is held together by the divalent calcium ions (23). Removal of calcium ions by chelating agents such as ethylenediaminetetraacetic acid (EDTA) results in the dissociation of C1 into its subcomponents with concomitant loss of activity (24). Activation of the classical pathway is characterized by a dependence on IgG or IgM antibodies complexed with antigens. The classical pathway also specifically requires the components C1, C2, and C4 as well as the divalent cations calcium and magnesium. Although the components C3 and C5 through C9 are usually considered part of the classical system, they are shared by the alternative pathway and thus are not considered as unique components of the classical system per se.

The recognition and initiation function with respect to immunoglobulins resides with the C1q subcomponent (25,26). C1q itself is a rather peculiar protein consisting of three different polypeptide chains (27). Chemically, C1q contains approximately 10% carbohydrate, 5%

hydroxyproline, 2% hydroxylysine and 18% glycine. This unusual collagen-like composition makes it unlike any plasma protein yet described (28,29).

When complement is activated by antibody-antigen complexes such as exists on the surface of an antibody sensitized erythrocyte (EA), it undergoes a self assembly process sequentially depositing the entire fluid phase cascade onto the surface of the target. Specifically, C1q recognizes a previously sequestered binding site located in the Fc fragment of IgG and IgM (30,31). The three polypeptide chains of C1q are physically arranged in a manner perhaps analagous to a six headed mace or bola with each "head" representing a binding site for an IgG molecule (32). Thus each C1q molecule has six binding sites for IgG (and presumably the same number for IgM). Internal activation of C1 probably is the result of a conformational change in C1q which in turn induces a change in the proenzyme C1r (33). Once C1r is activated to $\overline{C1r}$ it is endowed with enzymatic activity through which the proenzyme C1s is converted to C1 esterase($\overline{C1s}$)(34,35,36). $\overline{C1s}$ is a serine esterase and is inhibited by diisopropylphosphofluoridate (DFP) (37). This esterase activity can be used to hydrolyze the synthetic substrates p-Tosyl-larginine methylester (TAMe) and N-acetyl-l-tyrosine ethylester (ATEe) (38). Recently, Loos and Raepple have demonstrated that many polyanions were capable of inhibiting the activity of C1 either by interfering with C1q binding to the antibody-antigen complex, or by preventing interaction of C4 and C2 with $\overline{C1s}$ (39,40). Although binding of C1 usually leads to activation, the two processes are not integral--IgG with modified tryptophan (41) and the human immunoglobulin subclass IgG4 (42)--both bind C1q but do not activate C1.

After activation, $\overline{C1s}$ enzymatically cleaves C4 into a large (C4b)

and small fragment (C4a) (43). The cleavage of C4 exposes a membrane attachment site on the C4b molecule and it will attach to the antibody-antigen complex at a site juxtaposed to the C1-antibody complex (44,45). $\overline{C1s}$ then cleaves C2 into C2a and C2b (46) with C2a attaching to the C4b site and C2b being released into the fluid phase. Thus, the molecular complex $\overline{C4b2a}$ is formed and is referred to as C3 convertase because it is capable of splitting and activating C3 (47,48). C3 convertase is also an esterase, and although C3 is its natural substrate, it also hydrolyzes the ester bond of acetyl-glycl-lysine methyl ester (49). The catalytic site of C3 convertase is believed to reside in the C2a subunit and even after release from the C4b complex, cytolytically inactive C2a retains esterase activity, but is no longer capable of cleaving C3 (49). The enzymatic half-life of $\overline{C4b2a}$ is quite ephemeral--only 10 minutes at 37°. However, if the C2 is first oxidized by treatment with iodine (applicable to human but not guinea pig C2), not only is the binding of C2a to C4b enhanced, but the half life of the bimolecular complex is increased 20 fold (50). No doubt the transient association of C2a with the $\overline{C42}$ and $\overline{C423}$ complex plays a vital role in controlling the complement reaction by temporarily limiting the functional association of these complex enzymes.

Once C3 is cleaved into C3a and C3b, the small C3a fragment is released into the fluid phase and C3b becomes associated with the $\overline{C4b2a}$ complex and with other non-hemolytic sites on the target membrane (47). The association of C3b with the C3 convertase modulates its activity so that now C5 becomes the natural substrate of this trimolecular complex. The $\overline{C423b}$ complex is referred to as C5 convertase (51) and like $\overline{C42}$, is a highly specialized protease. Just as C3 is the only known protein

substrate for $\overline{C42}$, C5 is the only known substrate for $\overline{C423}$.

Once C5 is cleaved into C5a and C5b, C5a is released in the fluid phase and C5b transiently acquires the ability to bind one molecule each of C6 and C7 (52,53). With this, a self-assembly process is initiated and results, without any further enzymatic activity, in the formation of the stable C5b-9 complex (54). It should be noted that the small by-product fragments C3a and C5a are endowed with marked phlogogenic activity (55,56,57). Some of these activities include release of histamine from mast cells, contraction of smooth muscle tissue, directed chemotaxis of polymorphonuclear leukocytes, and vasodilation both in conjunction and independent of histamine activity (58). Such potent pharmacological activities obviously play a major role in the normal course of the inflammatory response.

Once the $\overline{C5b67}$ complex is formed, it too can bind nonspecifically to areas on the membrane other than at the location of the C5 convertase (52). The trimolecular association of $\overline{C567}$ provides the molecular arrangement for the adsorptive binding of one molecule of C8 which in turn provides a binding region for up to six molecules of C9 (54). A low grade lesion of the target membrane occurs with only the addition of C8 to the complex (59); but with the binding of C9, a ten component macromolecular complex is formed which greatly enhances the rate of target cell cytolysis (54). It should be noted that the $\overline{C5b67}$ complex or even the $\overline{C5b67}^{C3}$ complex can attach to non-sensitized "innocent by-stander" cells and thus promote a terminal cytolytic event. This phenomenon has been termed "reactive lysis" (60) and is controlled by the rapid decay of the unbound complex (61,62).

The precise mechanism by which complement mediates cytolysis of

susceptible target cells is not clearly understood. One hypothesis, in light of the newly discovered tributyrinase activity of C7, is that the lytic event is caused by an enzymatic attack on the membrane (63). However, no enzymatic degradation products have ever been discovered in either lysed cell membranes or in ruptured synthetic lipid bilayers (64). The two most favored models are the "doughnut" insertion hypothesis (65) and the C8 insertion model (29). The former model purports that the C5b-9 complex inserts inself into the membrane as a "prefabricated hole" allowing the exchange of intra- and extracellular material via an internal hydrophilic channel (65). However, the model fails to explain how the hydrophilic complement components enter the hydrophobic expanses of the membrane. In addition, although electron microscopy has revealed apparent ultrastructure doughnut shaped "lesions" on the surface of cells lysed by complement (66), freeze etching techniques have shown that the ultrastructure alterations are confined to the outer leaflet of the membrane, i.e. the lesion does not penetrate the membrane (67). The C8 insertion model embraces most of the salient features of the doughnut model, but in addition postulates that the α and γ chains of C8 are inserted into the channel formed by the surface macromolecular complex. The α and γ chains thus extend into the membrane bilayer causing disruption of orderly structure.

In addition to the restraints placed on the complement cascade due to the rapid decay of several of the intermediates, there are two naturally occurring inhibitors of complement present in the sera of man and probably in all vertebrates. The first inhibitor is referred to as $\overline{C1s}$ inhibitor and, as the name implies, it directly abrogates the hemolytic and esterolytic activity of C1 (68,69). The second inhibitor is

referred to as C3b inactivator and cleaves both soluble and cell bound C3b into two antigenically distinct fragments, C3c and C3d (70). As a result, $\overline{C423}$ loses C5 convertase activity, and C3b activation of both the alternative pathway and the immune adherence phenomenon is abolished (71,72,73). This latter activity can be visualized by the clustering of cells bearing C3b on their surface around other cells displaying C3b receptors. Such receptors have been shown to be present on human erythrocytes, polymorphonuclear leukocytes, platelets, macrophages, and on B lymphocytes (74,75). The attachment of C3b not only plays a direct role in the increased opsonization of target cells (76), but C3b binding to B lymphocytes has been postulated to play a role in B-cell activation as well (77).

The second pathway by which complement may be activated is referred to as the alternative or properdin pathway. Historically, the existence of this pathway had been suggested as early as 1954. At that time, Pillemer and his associates reported the discovery of a new protein in normal human sera (78). Properdin, as it was called, was capable of reacting non-specifically with diverse naturally occurring polysaccharides and lipopolysaccharides ultimately resulting in the activation of complement. This process ostensibly occurred without the interaction of antibody and was proposed as a major pathway by which susceptible bacteria and viruses were destroyed. However, this provocative hypothesis was discarded as apocryphal and the described activities were attributed to the presence of natural antibodies (79). The controversy remained unresolved until recent years when rigorous immunochemical techniques were employed in the isolation, purification, and determination of function of many of these components. The unanticipated complexity of the properdin

system has spawned a multiplicity of models attempting to elucidate its precise mode of initiation and function. Clearly, a plethra of diverse stimuli are capable of activating this pathway, and this fact alone imposes a formidable constraint on any molecular model. Some of the more common naturally occurring activators of the alternative pathway include bacterial and fungal cell wall constituents such as lipopolysaccharide, zymosan, and inulin (a polyfructose) (71,80-83). In addition, aggregates of some immunoglobulin classes (84,85), some types of animal cell membrane constituents (86,87), and antibody-coated budding virus infected cells (88,89) also stimulate this pathway. The alternative pathway can even be activated by substances of relatively defined chemical nature such as benzyl-B-D-fructopyranoside (90), polyglucose with repititious α 1-3 and branched α 1-6 linkages (91), dinitrophenylated albumin (92), and many polyanionic substances. Cobra venom factor (a non-lipolytic, non-hemolytic glycoprotein isolated from the venom of the cobra Naga naja) is also a potent activator of complement cytolytic potential, but it appears to act as a C3b analog and is thus unique in its mode of alternative pathway activation (93,94,95). Potentiation of this system requires devalent magnesium ions and the interaction of at least five novel serum proteins. By convention, the names of these proteins are IF (or initiating factor), P or $\bar{P}$ (properdin). Factor B (C3 proactivator), Factor $\bar{B}$ (C3 activator), and Factor D or $\bar{D}$ (C3 proactivator convertase). To date, all of the above components have been isolated, purified, and characterized as to molecular weight, electrophoretic mobility, and sedimentation coefficients (83,96-98). C3b (of the classical pathway) plays an intregal role in the alternative pathway (71,96,99), and thus it in essence forms the junction point of the two systems. Because all terminal

components (C3, C5-9) are shared, the biological consequences of activation encompass all the processes previously described (immune adherence, opsonic activity, anaphylatoxin production, membrane attack complexes, etc.).

There are similarities between some of the more salient features of the classical pathway compared with those of the alternative pathway. Analogous to C1q, IF seems to function as the recognition unit for the properdin pathway, but its relationship to another factor (referred to as a C3 nephritic factor from the sera of patients with membranoproliferative glomerulonephritis (100) and its mode of activation is poorly understood (96). Factor $\overline{D}$ is capable of enzymatically cleaving Factor B into Ba and $\overline{Bb}$ (29,94). In the presence of C3b, a bimolecular complex $C\overline{3b}\overline{Bb}$ is formed (29) which is endowed with C3 splitting activity similar to the C3 convertase ($C\overline{4b2a}$) of the classical pathway. Furthermore, just as C4b anchors the classical convertase to the membrane allowing C2a to exert its enzymatic activity, so too cytophilic C3b anchors the $C\overline{3b}\overline{Bb}$ complex to the membrane allowing the enzymatic activity of Factor Bb to be expressed (83). Both complexes merely gain additional C3b to modulate C5 cleaving activity (99). Thus, the presence of C3 not only prevents an "abort" due to rapid decay of either convertase, but because C3b is utilized as part of the alternative pathway convertase, it participates in a type of amplification loop. In other words, the more C3b that is formed from either pathway, the more C3 cleaving potential is endowed upon the properdin C3 convertase. Properdin (P) seems to stabilize the fragile $C\overline{3b}\overline{Bb}$ complex but its possible role in stabilizing the classical C3 convertase has not been investigated (99). Noteworthy, however, is the potent effect properdin exerts on the C3b inhibitor (99). By

modulating the action of this enzyme, properdin at least indirectly plays a role in stabilizing the classical pathway sequence.

The recognition of foreign substances by a host usually leads to the neutralization and eradication of these substances by immune lymphocytes, phagocytic cells, specific antibodies, complement, or an amalgamation of these factors. However, in instances where antigenic substances interact directly with host tissue, the reactions of the host's immunological defense system could sometimes result in a considerable amount of autodestruction. LTA represents a class of antigens that are capable of spontaneous cytophilic binding to mammalian tissue (101,102,103). As a result, host tissue acquires a new "anitgenic face" and may now react with natural or induced antibodies to the LTA. Furthermore, antibodies directed primarily at LTA determinants may cross react with similar determinants of the host's tissue. Such a mechanism has been proposed for the high incidence of rheumatic fever and glomerulonephritis in patients recovering from post streptococcal infections (104,105). Recently, acylated heteropolysaccharides (LTA) isolated from the cell membranes of several lactobacillus species were shown to replace pigeon excreta antigens in complement consumption tests diagnostic for pigeon breeders disease (9,106). Thus, precedence may already be established for LTA's role in the manifestation of several clinical maladies. In addition, the chemical and biological similarities between LTA and LPS (1,2) plus the ability of LTA to stimulate bone resorbtion (17) make LTA a likely candidate for a role in periodontal disease. On the other hand, LTA lacks some of the biological activities associated with LPS such as pyrogenicity in rabbits (2,107) and a mitogenic effect on B-cells (2). Since these activities have been shown to reside with the complex

Lipid A of LPS (108,109) and since the unique sugars and hydroxyacyl esters of Lipid A are absent in LTA, it is not surprising that associated activities are absent as well. As a class, teichoic and lipoteichoic acids are wall and membrane components of gram positive bacteria (107,108). LTA is typically membrane associated and consists of a glycolipid covalently linked to a polyglycerolphosphate backbone which may carry carbohydrate and D-alanine substituents (2). Teichoic acids (TA), however, are never associated with cell membranes; they lack the terminal glycolipid coupling, and they may have a backbone of either polyglycerolphosphate or polyribitol phosphate (2). LTA may be converted functionally to polyglycerol TA by spontaneous deacylation in an aqueous environment,[1] or mild alkaline, or acidic hydrolysis (107). The molecular weight of LTA (93) is probably between 3000-12000 but because of its tendency to form micelles in an aqueous environment, the apparent molecular weight as determined by gel filtration is approximately four million (110).[2] Because LTA possess the glycolipid moiety, they are amphipathic molecules exhibiting a propensity to spontaneously associate with proteins and biological membranes (103). Mammalian red blood cells can be "coated" by spontaneous adsorbtion with an LTA containing extract and the cells can subsequently be agglutinated with an anti-LTA serum. Passive hemagglutination (PHA) performed in this manner with sheep red blood cells has previously been reported by many investigators who discovered

[1] Personal communications from R. Craig, Dept. of MCS, Univ. of Fl.; K. Knox and A. J. Wicken, Institute for Dental Research, Sydney, Australia; and personal unpublished data.

[2] Data supported by personal experience (see Figures 11 and 12), and personal communication from R. Craig.

erythrocyte-sensitizing antigens in cell free saline washings or spent culture fluid from several gram positive organisms (101,102). These so called "Rantz antigens" were recently shown to possess properties associated with LTA (111). Because only acylated LTA will bind to erythrocytes, PHA provides a means of quantitating the amount of LTA in a preparation without having to contend with deacylated TA contamination.

The biological role of TA and LTA to the microorganism has been a subject of considerable disputation by several investigators in recent years. Thus far, at least three roles have been tentatively assigned: 1). TA and LTA seem to function as "carrier" molecules for membrane and cell wall components, i.e. amphipathic LTA may be used by the cell to transport needed hydrophobic molecules through hydrophilic zones which would otherwise pose an almost impenetrable barrier. Fielder and Glaser have established that intracellular LTA serves as a lipid carrier for the biosynthesis of cell wall ribitol teichoic acid in Staphylococcus aureus (112,113). Chaterjee and Wong (114) have demonstrated that LTA may serve as the acceptor in which nascent peptidoglycan polymers are synthesized. 2). LTA seems to be involved in cell wall division and regulation. Holtje and Tomasz have reported that LTA exhibits an inhibitory effect on the function of autolytic enzymes during the division cycle of pneumococcus (115). It is interesting to note that similar functions have been described by Cleveland, et al. working with a strain of Streptococcus faecalis (116,117,118). In these systems, LTA is deacylated and released into the environment as TA. Once the concentration of LTA is sufficiently lowered, or the concentration of autolytic enzymes is sufficiently elevated, cell wall autolysis begins at the division zone. This autolytic activity then allows for insertion of additional

cell wall material. 3). LTA or TA may contribute to the overall electrostatic charge of gram positive organisms. Although membrane localized, the long polar tails of many LTA penetrate the thick peptidoglycan layer and become externalized (107). These, together with the TA which are covalently linked to the cell wall (108) present a myriad of antigenic faces to the external environment (119,120). This antigenic presentation is of serological import since these antigens are often genus, species, group, or type specific (103,120). In addition, these polar tails generate a net negative charge by exposing the phosphate groups of the polyglycerol or polyribitol backbone. This net negative charge has been teleologically assigned the function of maintaining electrostatic repulsion and dispersion of the bacterial cell (121). Since LTA has been shown to sequester certain cations such as magnesium (122), an additional function as a site of divalent cationic convergence has also been postulated. The association with magnesium ions appears to be more than casual since protoplasts of Lactobacillus casei placed in a magnesium ion free or chelated medium rapidly lose their LTA from the cell membrane.

Anti-LTA titers (of both the IgM and IgG classes) have been regularly reported in mice, rabbits, and man (2,123). Several clinical studies have reported increases in anti-LTA titer--including antibodies of the class IgA--after acute gram positive infections (124,125). Pigs, guinea pigs, and rats exhibit a low level of natural immunity to LTA and recently, there have been reports of salivary IgA production as a result of gastric intubation of monkeys with Streptococcus mutans 6715 serotype C. There is no doubt that TA and LTA of all gram positive genera thus far investigated contain antigenic moieties and that under certain circumstances

LTA can be immunogenic (2). Of particular interest is the fact that the attachment of streptococcal LTA to erythrocytes could be reversibly transferred from the erythrocytes to other tissue cells (104,126). The possible significance of this "transferability" in relation to rheumatic fever and glomerulonephritis and pigeon breeders disease has been previously discussed (9,104-106). However, despite this precedence the significance of the binding of LTA to oral epithelial cells in gingival pockets has not yet been investigated. Not only does LTA mediate bone resorbtion as previously indicated, but spontaneous hybrid micells of LPS and LTA are known to occur,[1] thus compounding the possibility of *in situ* immunological modulation. There is little doubt of the availability of extracellular LTA in this environment--*Streptococcus mutans* BHT alone has been reported to produce excess of 30 µg of LTA/ml in culture media (20). Recently, Wicken and Knox have studied the excretion of extracellular LTA from this organism in a chemostat under steady state logarithmic growth conditions. Results indicated that a generation time of 10-14 hours (estimated to reflect that actual *in vivo* growth rate of this organism in the oral cavity) produced the maximal amount of extracellular LTA (1). Considering its ubiquity and the cariogenic nature of *Streptococcus mutans* BHT (127-130), the secretion of copious amounts of biologically active LTA into the oral cavity has the potential of considerable influence on the host-parasite relationship.

The objectives of the project were then defined as follows:

(1) To establish if an LTA-containing extracellular extract of

[1] Personal communication of A. J. Wicken.

Streptococcus mutans BHT was capable of inhibiting complement mediated cytolysis of target sheep erythrocytes.

(2) To purify the extracellular LTA of S. mutans BHT to homogenity.

(3) To describe the nature of any anti-complementary activity that purified extracellular LTA may exhibit.

(4) To determine the site of action of any such inhibition.

(5) To determine the mechanism by which purified extracellular LTA may enhibit anti-complementary activity.

(6) To determine if the LTA from other gram positive genera and species can be shown to demonstrate anti-complementary activity.

MATERIALS AND METHODS

Crude extracellular LTA (LTAcx). The initial studies were carried out utilizing LTAcx prepared in Australia by the method of Wicken and Knox (110). Streptococcus mutans BHT was grown to late stationary phase in a New Brunswick Microfirm fermentor at 37°C, under anerobic conditions (95° N_2 and 5% CO_2) in a complex medium.

Later experiments utilized LTAcx prepared at Gainesville, Florida. The original method was modified as follows. A Pellicon Cassette system (Millipore Corp., Bedford, MA) equipped with 5.0 ft^2 of PTGC filter material was used to dialyze Todd-Hewitt broth (Difco Laboratories, Detroit, MI). A 100 ml culture of early log phase S. mutans BHT was inoculated into 10 liters of dialyzed medium and incubated at 37° for 24 hours. The cells were harvested using a Delaval Gyrotester (Poughkeepsie, NY). The supernate was passed through the Pellicon Cassette system (loaded with 1.0 ft^2 of 0.45 μ microporous membrane) to remove remaining cells and debris. The cell-free spent fluid was then fractionated and concentrated by passage through 5.0 ft^2 PTGC membrane (nominal molecular weight exclusion limit of 10,000). The filter retentate was washed in situ with several liters of water, collected and lyophilized. The freeze-dried retentate, designated as LTAcx, was stored in a dessicator at -20°C.

Solutions for complement assays. Isotonic Veronal buffered sodium chloride (VBS), dextrose gelatin Veronal buffer with added

$CaCl_2$ and $MgCl_2$ (DGVB), EDTA containing Veronal buffer (0.04 M EDTA-GVB) and gelatin Veronal buffer with added $CaCl_2$ and $MgCl_2$ (GVB) were prepared as previously described by Hoffmann (131).

Human complement (HuC). Fresh human blood samples were obtained from the Gainesville Plasma Corp., Gainesville, FL. The blood was allowed to clot at room temperature for about 60 minutes, and the serum was separated by centrifugation at 500 X g at 0°C. The serum was collected and stored at -70°C.

Guinea pig complement (GPC). Fresh frozen guinea pig complement was purchased from Pel Freeze Laboratories (Rogers, AR). The serum was shipped in dry ice and it was stored at -70°C after arrival in the laboratory.

Complement components. Purified guinea pig C1 and C2 were prepared according to Nelson et al. (132) and Ruddy and Austin (133,134). Functionally purified guinea pig C3, C8 and C9 and human C1, C5, C6 and C7 were purchased from Cordis Laboratories (Miami, FL).

Erythrocytes (E). Sheep blood was taken by venipuncture from a single animal maintained at the animal research laboratory of the J. Hillis Miller Health Center (Gainesville, FL). One hundred milliliter volumes of blood were collected in equal volumes of sterile Alsever's solution (135) and the blood was stored at 4°C for up to three weeks.

Antibody sensitized sheep erythrocytes (EA). Rabbit anti-sheep E stromata was obtained from Cordis Laboratories (Miami, FL). Sensitization of washed sheep E was performed as recommended by the supplier.

Complement component intermediate complexes. Sheep E in various stages of complement fixation were used in this study. $EAC\overline{1}$, $EAC\overline{14}$ and $EAC\overline{142}$ were prepared by methods described by Borsos and Rapp (136). $EAC\overline{1423567}$ were prepared by the procedure described by Hoffmann (137). Unless otherwise indicated, guinea pig C1, C8 and C9 were used in all instances, and the remaining C components were from human serum.

Treatment of cells and cellular intermediates with LTAcx. Unless otherwise indicated, cells were washed and suspended in VBS at a concentration of 10^9/ml. Equal volumes of these cells and LTAcx were mixed and incubated at 37° for 20 minutes with continuous shaking. The mixture was then placed in an ice bath for 10 minutes. At the end of incubation DGVB was added to the mixture and it was centrifuged at 500 g for five minutes. The supernate was discarded and the cells were suspended and washed thrice with DGVB (0° for 10 minutes at 500 g) to remove any unbound material. The cells were then resuspended in DGVB at a concentration of 10^8/ml. A sample of the cells were tested for cell-bound LTA using passive hemagglutination with rabbit anti-LTA. The remaining cells were used in experiments to detect acquired resistance to hemolysis.

Passive hemagglutination (PHA). Passive hemagglutination was carried out using a microtitration system. Fifty ul of a VBS dilution of anti-LTA were added to the first row of wells of a round bottom microtiter plate (Cook Engineering Co., Alexandria, VA) and 25 ul (one drop from the calibrated pipetes supplied with the system) of VBS were added to the other wells on the plate. The anti-serum was serially diluted *in situ* and one drop of LTAcx

treated cells was added to each well. Controls for spontaneous or nonspecific agglutination consisted of wells that contained antiserum and sheep E which had never been exposed to LTAex. Treated sheep E plus VBS constituted another control. The microtiter plate was incubated at 37°C on a Cordis Micromixer (Cordis Laboratories, Miami, FL) for 15 minutes. The plates were removed from the mixer and the cells were allowed to settle for two hours at 37°C, followed by three hours at room temperature.

Modified passive hemagglutination (PHAg). A modification of the above technique was used to semi-quantitate the amounts of LTA present in various preparations. The same apparati were used, but instead of antibody, LTA-containing extracts were added to the bottom wells and serially diluted in situ as described. After each LTA source was diluted, one drop of sheep erythrocytes (10^8/ml in VBS) was added to each well and the plate was then incubated at 37°C for 20 minutes and at 0°C for 10 minutes. The cells were kept in suspension by vibrating the plate on a Cordis Micromixer during both incubation periods. One drop of GVB was then added to each well and the plate was centrifuged at 200 g for 5 minutes. The entire plate was then abruptly inverted over absorbant paper towels and allowed to drain for approximately one minute. One drop of GVB was again added to each well and the plate was vibrated at 0°C for 5 minutes to resuspend the pellet. An additional drop of GVB was added per well and the plate was again centrifuged at 200 g for 5 minutes. This washing procedure was repeated three times and the cells were then finally resuspended in one drop of GVB. One drop of anti-LTA (diluted 1:1000 in VBS) was then

added to each well and the plate was incubated at 37°C for 15 minutes on a Cordis Micromixer. The plate was removed from the mixer and the cells were allowed to settle for two hours at 37°C, followed by three hours at room temperature.

Inhibition of complement mediated lysis. EA coated with LTA (EA_{LTA}) were tested by mixing 0.1 ml of EA_{LTA} (10^8 cells/ml) in DGVB and 0.4 ml of DGVB diluted HuC. The HuC was diluted so that a maximum of 80 percent lysis was produced in EA which had not been treated with LTAcx. The mixture was incubated at 37°C with continuous shaking for 60 minutes. One milliliter of ice cold EDTA-GVB was added, the mixture was centrifuged for 5 minutes at 500 g at 0°C and the supernatent fluid was recovered. The optical density of the supernatent fluid was determined at a wave length of 414 nm. Inhibition of hemolysis was calculated for each concentration of LTAcx used by comparing the extent of lysis in each assay with a control reaction mixture which contained EA that had not been treated with LTAcx.

Effect of LTAcx on the titer of antibodies specific for sheep erythrocyte stromata. Because LTA associate with some proteins (138) it was necessary to perform a hemolytic antibody titration to determine if the ability of the immunoglobulins to fix complement at the cell surface was being affected by LTAcx treatment. The possibility of similar antigens in LTAcx and sheep erythrocyte stromata was also considered. Equal volumes of LTAcx (500 ug/ml in VBS) and rabbit anti-sheep E stromata were incubated together at 37°C for 20 minutes. A control consisted of incubating an

equal volume mixture of VBS and anti-sheep erythrocyte stromata for the same time at the same temperature. The antibodies were then titrated using limiting amounts of complement (135).

Cl fixation and transfer. The number of $\bar{C}l$ molecules bound to an antigen-antibody complex can be measured by the Cl fixation and transfer test described by Borsos and Rapp (139). In a modification of this procedure, an attempt was made to quantitate the number of $\bar{C}l$ molecules fixed to EA which had previously been treated with LTApcx. Buffer controls and EA_{LTA} were prepared as previously described, and after washing were resuspended at 10^8 cells/ml in DGVB. Equal volumes of EA_{LTA} and EA_{VBS} were incubated with $\bar{C}l$ at 30°C for 15 minutes. The cell mixtures were washed twice with DGVB, and resuspended in GVB at a cell concentration of 1 X 10^8/ml, 5 X 10^7/ml, and 1 X 10^7/ml. One volume of each cell concentration was added to one volume of EAC4 (at 1 X 10^8 cells/ml) to permit transfer of $\bar{C}l$ from $EA_X\bar{C}l$ [1] to EAC4. The cells were incubated at 30°C for 15 minutes, and then C2 and C-EDTA were added in relative excess as described previously.

A variation of the $\bar{C}l$ transfer assay was performed by treating preformed $EA\bar{C}l$ with LTA or buffer control as described. The resulting $EA\bar{C}l_x$ were resuspended to 1 X 10^8 cells/ml in GVB and the amount of $\bar{C}l$ capable of transfer was measured as described above.

Gel filtration. LTAcx was fractionated on a 2.5 cm X 100.0 cm column of Bio-Gel A-5M, 200-400 mesh (Bio-Rad Laboratories, Richmond,

[1] In this instance, "x" represents LTA or the appropriate buffer treated control.

CA) using a modification of the method described by Wicken and Knox (110). The column was equilibrated and eluted using 0.01 M Tris carbonate (Sigma Chemical Co., St. Louis, MO), pH 6.8.

Hydrophobic Affinity Column chromatography.[1] Because of the hydrophobic nature of the fatty acid moieties of lipoteichoic acid, adsorbtion to a stationary phase of a chromatographic column was used in an attempt to further purify the LTA. LTAppx in buffer A (0.01 M Tris carbonate pH 6.8, 1.0 M Nacl was loaded on a 25.0 X 2.25 cm column packed with Octyl Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) and equilibrated in the same buffer. After eluting with 150 ml of starting buffer A, the reservoir was then changed to buffer B (0.01 M Tris carbonate pH 6.8) and another 100 ml were eluted. Buffer C consisted of 250 ml of a gradient ranging from 10-70 % propanol (by volume) in 0.01 M Tris carbonate, pH 6.8.

Octyl Sepharose is a derivative of the cross linked agarose Sepharose CL-4B. The terminal n-octyl groups of this agarose gel confer a hydrophobicity to the matrix. By exploiting this property it was hoped that polar or neutral non-interacting components would be removed by elution with solutions of high ionic strength. The lipoteichoic acid would then be eluted from the matrix with an organic solvent such as propanol. (It is imperative that all tubing, connections and gaskets used throughout the column be constructed of a material that is resistant to organic solvents).

[1]This method represents a modification of a procedure described by A.J. Wicken and K. Knox (Sydney, Australia) via personal communication.

Removal of salt and propanol from LTA containing extracts. Removal of salts and/or propanol from various preparations was rapidly and quantitatively accomplished by gel filtration utilizing LH20 (Pharmacia Fine Chemicals, Piscatawav, NJ) as the solid phase support matrix. The most commonly employed column was 50.0 cm X 2.5 cm but a larger 65.0 cm X 3.0 cm column was sometimes utilized. The column was packed and equilibrated with deionized water. Sample preparations usually involved rotary flash-evaporation (Buchler Instruments, Fort Lee, NJ) in order to reduce the volume of sample to 15-20 ml. Elution of product was carried out at a pressure head of approximately 50 cm water and approximately 4.0 ml effluent were collected per tube.

Phosphatidyl choline vesicle (PCV) purification of LTA -- (a) Preparation of PCV. Although reported as the method of choice by other investigators,[1] in our hands Octyl Sepharose purification of LTA from Streptococcus mutans BHT resulted in a product still highly contaminated with polysaccharides. In an attempt to achieve homogeneous purification of LTA, a modification of the above mentioned hydrophobic adsorbtion principle was employed. In this procedure, artificial membrane vesicles were prepared with DL-phosphatidyl choline dipalmitoyl (PC) (Sigma Chemical Co.) as the sole constituent via a modified method of Hill (140). In brief, 40.0 mg of PC was placed in each of several 30 ml high speed glass Corex centrifuge tubes (Corning Glass Works, Corning, NY) and dissolved with one ml chloroform. The solvent was gently evaporated in a 50°C water bath while rotating the tubes so as to coat the bottom 5 or 6 cm of the tube with PC. Once dry, the tubes were placed in a lyophilization flask and any residual solvent was removed in vacuo. One milliliter

[1]Wichen, A.J., and Knox, K.--Personal communication.

of 0.01 M Tris carbonate pH 6.8 was then added to each tube and they were placed in a 50°C water bath. Once warmed, the tubes were vigorously vortexed (Vortex Genie Mixer, Scientific Industries Inc., Bohemia, NY) and the cycle of warming and vortexing was continued until a milky emulsion was formed. Fifteen milliliters of 0.01 M Tris carbonate were then added to each tube and the tubes were centrifuged at 27,000 g for 30 minutes. The supernatent fluids were then decanted, the pellets were resuspended in 1.0 ml Tris carbonate buffer and warmed to 50°C in a water bath. The tubes were gently swirled (but not agitated) to dissolve and resuspend the pellet . The resulting phosphatidyl choline vesicles (PCV), devoid of very small vesicles, were then used to adsorb LTA from LTAppx.

(b) Preparation of PCV-LTA. Two milliliters of LTAppx at a concentration of 1.5 mg/ml in 0.01 M Tris carbonate, pH 6.8 were added to each centrifuge tube containing 1.0 ml of PCV. The tubes were covered with parafilm (American Can Co., Neehaw, WS) and incubated for 90 minutes in a 37° shaker water bath. Thirteen milliliters of 0.01 M Tris carbonate were then added to each test tube and they were centrifuged at 27,000 g for 45 minutes. The supernates were discarded and the pellets were gently resuspended in 1.0 ml of Tris carbonate buffer at 50°C as previously described.

Fifteen milliliters of buffer were then added to each pellet, the tubes were gently swirled and then centrifuged as described. The pellets were washed three times in this manner. The final pellet was drained and then dissolved in 5.0 ml of chloroform/methanol (3 + 1 v/v). The tubes were then covered with aluminum foil and allowed to sit at room temperature for 60 minutes.

A Millipore 15 ml analytical filter holder (Millipore Corp., Bedford, MA) was loaded with a 3.0 μ fluoropore membrane (Millipore Corp.) and washed with several volumes of the chloroform/methanol solvent. The test tubes were all sequentially decanted into the apparatus and the contents were allowed to filter by gravity through the membrane. Each test tube was washed with several volumes of warmed chloroform and decanted into the filtering apparatus. Finally, the barrel and filter were washed in situ with warm chloroform. The filter was removed after air drying in situ and placed in 10.0 ml of deionized water warmed to approximately 40°C. All centrifuge tubes and the barrel of the filtering apparatus were washed with warm deionized water and all products were combined. The resulting product was passed through a 25 mm Swinnex filter (Millipore Corp.) loaded with a 5 μ microporous membrane (Millipore Corp.) to remove particulate debris. The membrane was washed in situ with several volumes of warm deionized water. The filtrate was collected directly into a lyophilization flask and was then shell frozen and lyophilized. The final product was stored in a dessicator at -20°C.

^{14}C Phosphatidyl choline analysis. In order to detect any phospholipid contamination of the LTA throughout the previously described PCV purification, radioactive PC was used to label the phospholipids in the vesicles. Approximately 2.3 μCi (5×10^6 DPM) of ^{14}C labeled phosphatidyl choline (Amersham Searle Corp., Arlington Heights, IL) were added to 40 mg of phosphatidyl choline dipalmitoyl in a 30 ml Corex centrifuge tube. Phosphatidyl choline vesicles were prepared from this and the non-labeled contents of three other tubes by the methods previously described. Fifty microliter

samples from the ^{14}C containing test tube were taken at each step of the purification and placed in empty glass scintillation vials. The samples were heated to 50°C in a drying oven to remove the solvent from the sample. Once dry, 50 ul of chloroform were used to redissolve all samples and then 5.0 ml scintillation fluid containing toluene (scintillation grade, Mallinckrodt, St. Louis, MO), 0.4% PPO (2,5 diphenyloxazole), and 0.01% POPOP (1,4-di (2-(5-phenyloxazolyl)-benzene) were added to each vial. The degree of ^{14}C-PCV contamination of the final product was determined by placing the entire LTA-containing-fluoropore filter in a scintillation vial with 5.0 ml scintillation fluid. The possible influence of quenching by the fluoropore filter was investigated by adding equal aliquots of ^{14}C-PC to two scintillation vials one of which contained a fluoropore filter in addition to scintillation fluid. No appreciable difference in CPM was observed. Disintegrations per minute (DPM) values were calculated from a standard quench curve constructed for use with chloroform. Standard ratios were determined for each sample and percent efficiencies were extrapolated from the standard quench curve. This volume was then used to correct counts per minute (CPM) to DPM. Unless otherwise indicated, the samples were counted for 10 minutes in a Beckman LS-733 liquid scintillation counter (Beckman Instruments, Fullerton, CA).

Colormetric assays. Phosphorous was determined by the method of Lowry et al. (141) with absorbancies measured at 820 nm. Total carbohydrate was measured by the phenol sulfuric acid assay as described by Dubois et al. (142). Total protein was performed on samples using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Rockville Center, NY). Samples and the standard curve were prepared following the manufacturer's recommendations.

Gas Liquid Chromatography. Carbohydrate analysis was performed after treatment of the samples with 1.0 N H_2SO_4 in sealed ampules for 8 hours at 105°C. Upon cooling, the seal was broken and exactly 0.2 ml of mannitol (either at 5.0 mg/ml or 1.0 mg/ml depending on carbohydrate concentration of the sample) was added as an internal standard. The contents of each vial were quantitatively transferred to 15 ml centrifuge tubes (Corning Glass Works) containing 0.5 g $BaCO_3$. Each centrifuge tube was heated in a boiling water bath and alternately vortexed until the pH approached neutrality as indicated by full-range pH paper (Micro Essential Laboratory, Brooklyn, NY). All tubes were centriufged at 500 g for 5 minutes and the supernates were removed and collected in appropriately labeled 13 mm screw cap tubes fitted with teflon lined lids. The centrifuge tubes containing $BaCO_3$ were washed once with one ml of deionized water and the supernates were appropriately pooled.

After lyophilization, the hydrolyzed carbohydrates were converted to trimethylsilyl ester (TMS) derivatives by the addition of 0.2 or 1.0 ml (depending upon carbohydrate concentration) of TRI SIL Z (Pierce Chemical Co.). Samples were warmed to approximately 60°C in a water bath for 15-30 minutes before use and assayed using a Packard 800 series gas chromatograph equipped with a flame ionization detector. The gas chromatographic column (153 cm X 4 cm) was packed with SE-40 ULTRAPHASE 3% on Chromosorb W (HP) 80/100 mesh matrix (Pierce Chemical Co., Rockford, IL). Column and detector temperatures were set at 160°C and 195°C respectively. The N_2 carrier gas was set at approximately 30 cc/minute.

Amino acid analysis. Amino acids and amino sugars were measured on a JEOL model JLC-6AH automated amino acid analyser (JEOL, Inc., Cranford, NJ). Sample hydrolysates were prepared as described by Grabar and Burtin (143).

Clq, Cls, and $\overline{Cls}$ purification. Highly purified human Clq was prepared from whole human sera by the method of Yonemasu and Stroud (144). Highly purified human $\overline{Cls}$ and Cls were prepared by a minor modification of the method described by Sakai and Stroud (35). For the final resolution step, Bio-Rad Cellex-D DEAE with binding capacity of 1.07 meq/g (Cellex-D, Bio-Rad Laboratories, Rockville Center, NY) was substituted for fibrous DEAE cellulose Whatman DE-23. The DEAE was washed and prepared according to the manufacturer's specifications. Final elution of the product was accomplished with the use of the same eluting buffer as described, but instead of a stepwise elution of the column, an ionic gradient from 0.2 - 0.4 RSC (relative sodium chloride concentration) was utilized.

Disc acrylamide gel electrophoresis of Clq, Cls and $\overline{Cls}$. This was carried out essentially as described by Yonemasu and Stroud (144) but without the use of sodium dodecyl sulfate (SDS).

$\overline{Cls}$ inhibition assays. The ability of $\overline{Cls}$ to consume C2 activity was assayed by a modification of the method described by Sakai and Stroud (35). Briefly, 0.1 ml of $\overline{Cls}$ (approximately 8.0×10^7 site forming units, SFU/ml) plus 0.1 ml LTApcx (100 μg/ml in DVB) were incubated at 30° for 15 minutes. One tenth milliliter of C2 was then added at a concentration of approximately 9.0×10^7 effective molecules/ml and incubated at 37°C for 30 minutes. At the end of the incubation, 9.7 ml cold DGVB were added to the mixture resulting in a 1:100 dilution of the C2. The C2 was then serially diluted and 0.1 ml aliquots from each dilution were added to 0.1 ml of

$EAC\overline{14}$ (10^8 cells/ml in DGVB). The mixture was incubated at 30°C for 10 minutes and cooled to 0°C in an ice bath for 2.0 minutes. Three tenths of a milliliter of C-EDTA (1:37.5 in 0.04 M EDTA-$GVB^{=}$) were then added to each test tube and the mixtures were incubated at 37°C for 60 minutes. At the end of the incubation period, 1.0 ml of cold EDTA-$GVB^{=}$ was added, the tubes were centrifuged, and the supernates read for release of oxyhemaglobin at a wave length of 414 nm. External controls consisted of C2 with no $\overline{C1s}$ nor LTApcx, C2 with $\overline{C1s}$ but not LTApcx, and C2 with LTApcx but no $\overline{C1s}$. The usual internal controls (spontaneous lysis, color correction, no C2, and total lysis) were included at all times. Results were expressed as percent inhibition of C2 consuming ability compared with a control containing only $\overline{C1s}$ and C2.

The ability of $\overline{C1s}$ to hydrolize the synthetic substrate p-Tosyl-l-arginine methylester (TAMe) was determined as described by Nagaki and Stroud (38). Inhibition assays were performed by incubating equal volumes of $\overline{C1s}$ (approximately 8.0×10^7 SFU/ml) and LTApcx (approximately 100 µg/ml) at 37° for 10 minutes. Residual $\overline{C1s}$ activity was then determined as described (38-40).

Clq inhibition assays. The effect of LTApcx on the ability of purified Clq to bind to antibody sensitized sheep erythrocytes was determined by methods described by Loos et al. (39) and Raepple et al. (40). Equal volumes of Clq (approximately 1.5×10^8 SFU/ml) and LTApcx (10 µg/ml) were incubated at 37° for 10 minutes. Residual Clq activity was then determined as indicated above.

RESULTS

Inhibition of whole human complement by crude extracellular lipoteichoic acid (LTAcx). To determine whether LTAcx had any effect on whole human complement, equal volumes of LTAcx and whole human complement were preincubated at 37°/30 minutes. After preincuabtion, the complement source was serially diluted in DGVB and the residual hemolytic activity was titrated. As shown in Figure 1, approximately 50% of the whole complement hemolytic activity (measured in CH_{50} units) was consumed. Furthermore, as seen in Figure 2, this consumption was dependent on the concentration of the LTAcx used.

Titration of complement components in whole human sera after treatment with LTAcx. One mechanism for fluid phase consumption of whole complement could have been the interaction of natural antibodies in the human sera with LTA or some other antigenic substance in the crude extract. The result would be the fixation of C1 and subsequent activation of C4 and C2 via classical pathway. Another explanation for decreased hemolytic activity could have been the activation of the alternative pathway in a manner analogous to LPS. To differentiate between these two modes of activation, individual component titrations were performed on human sera incubated with LTAcx. In addition, C3 titrations were carried out in the presence of ethyleneglycol-bis (β Amino Ethyl Ether) N,N tetraacetic acid (EGTA) and Mg ions. This chelating agent preferentially binds Ca ions (145,146), and by reinforcing the EGTA buffer with Mg ions one can effectively deplete the available Ca ions yet maintain relatively high levels of Mg ions. Thus, the Ca ion dependent classical pathway is blocked, but the alternative pathway can function relatively unimpaired (145,147).

Figure 1. Titration of whole human complement after incubation with crude extracellular lipoteichoic acid (LTAex). Symbols: (o) Non-treated control; (•) Serum treated with LTAex at 500 µg/ml.

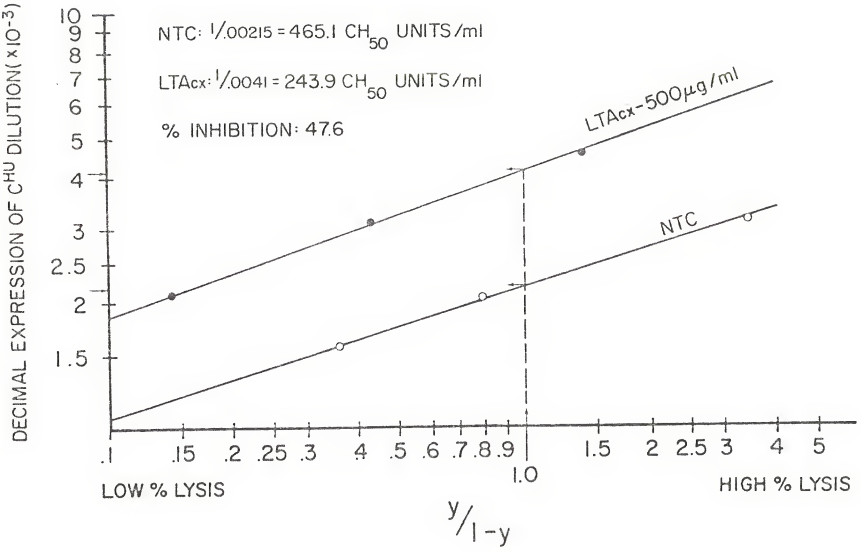
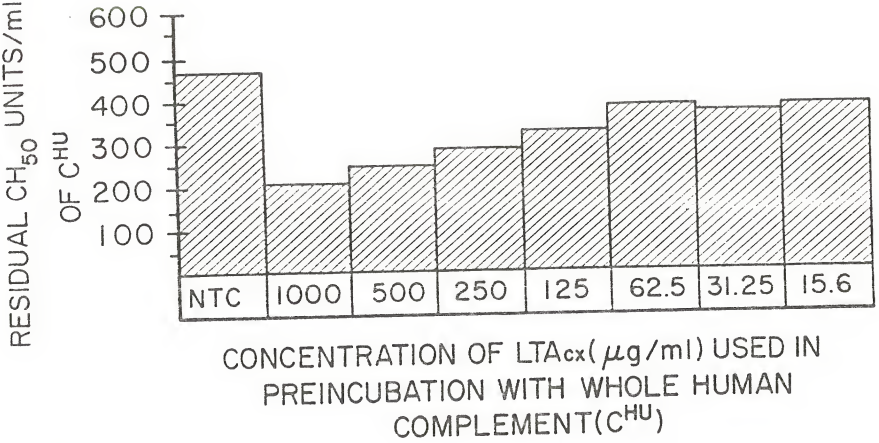

DECIMAL EXPRESSION OF C^{HU} DILUTION ($\times 10^{-3}$)
10
9
8
7
6
5
4
3
2.5
2
1.5
NTC: 1/.00215 = 465.1 CH_{50} UNITS/ml
LTAcx: 1/.0041 = 243.9 CH_{50} UNITS/ml
% INHIBITION: 47.6
LTAcx-500µg/ml
NTC
.1
.15
.2
.25
.3
.4
.5
.6
.7
.8
.9
1.0
1.5
2
2.5
3
4
5
LOW % LYSIS
HIGH % LYSIS
$y/1-y$

Figure 2. Dose response inhibition of whole human complement after incubation with varying concentrations of LTAcx. The non-treated control is abbreviated as NTC.


RESIDUAL CH_{50} UNITS/ml OF C^{HU}
600
500
400
300
200
100
NTC
1000
500
250
125
62.5
31.25
15.6
CONCENTRATION OF LTA_{cx} (μg/ml) USED IN PREINCUBATION WITH WHOLE HUMAN COMPLEMENT(C^{HU})

A typical component titration in serum treated with LTAcx is depicted in Figure 3. In this example, the LTAcx treated serum was serially diluted in DGVB. Next EAC$\overline{142}$, C5, 6, 7, and C8-9 were added sequentially to the dilutions. Since all components were added in excess, C3 became the limiting factor in contributing to the hemolysis of the target cells. Percent lysis in each test tube was mathematically converted to Z (the average number of SAC$\overline{1423}$ sites per cell) and this was plotted against the reciprocal of the serum dilution. Percent inhibition of site forming units (SFU) was then calculated from Z=1 values or percent inhibition of CH_{50} units was determined from values associated with Z= 0.69. Figure 4 represents a composite of multiple component titrations from whole human sera treated with LTAcx. As can be seen in this figure, C1 and C4 activities were consumed to some degree, however, more than 50% inhibition of C2 activity was observed. As indicated, C3 activity was also consumed during preincubation of complement with LTAcs, but incubation with purified $C3^{HU}$ produced no inhibition of C3 hemolytic potential. No C3 consumption occurred if the incubation was performed in the presence of the chelator ethylenediamine tetra acetic acid (EDTA) and less than 7% if incubated in the presence of EGTA-Mg ions. The above results indicated the necessity for divalent cations as cofactors mediating the consumption of C3 in the presence of LTAcx. In addition, there appeared to be a requirement for other serum factors (possibly natural AB and/or components of the alternative pathway) since purified C3 activity remained unaffected by incubation with LTAcx.

Figure 3. Titration of C3 in whole human serum after treatment with LTAcx. Symbols: (●) Non-treated control; (○) Serum treated with LTAcx at a concentration of 250 µg/ml. After incubation, sera were titrated for residual C3 activity according to procedures described in Materials and Methods.

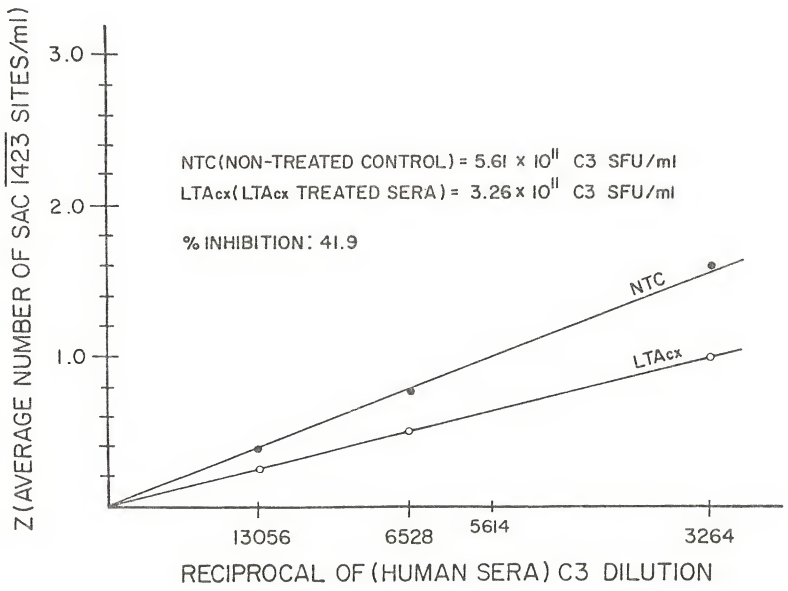
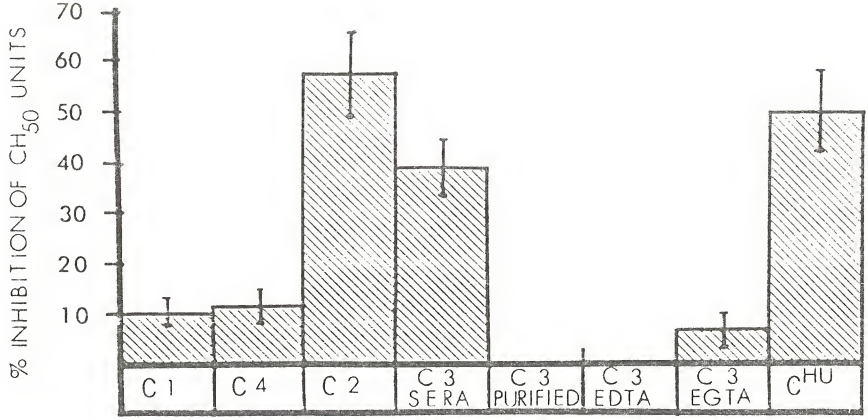

Z (AVERAGE NUMBER OF SAC $\overline{1423}$ SITES/ml)
3.0
2.0
1.0
NTC(NON-TREATED CONTROL) = 5.61 x 10^{11} C3 SFU/ml
LTAcx(LTAcx TREATED SERA) = 3.26 x 10^{11} C3 SFU/ml
% INHIBITION: 41.9
NTC
LTAcx
13056
6528
5614
3264
RECIPROCAL OF (HUMAN SERA) C3 DILUTION

Figure 4. Complement component titration of whole human sera after treatment with LTAcx. The sera were incubated with the LTAcx (500 ug/ml) then titrated for residual activity of the components indicated as described in Materials and Methods.


% INHIBITION OF CH_{50} UNITS
70
60
50
40
30
20
10
C 1
C 4
C 2
C 3 SERA
C 3 PURIFIED
C 3 EDTA
C 3 EGTA
C^{HU}

Inhibition of complement lysis of LTAcx treated EA. During an experiment in which EA treated with LTAcx were tested for reactive lysis, it was discovered that the LTAcx treated cells exhibited less hemolysis than even the buffer treated controls. This serendipitous observation led to the discovery that LTA treated EA were refractory to complement mediated lysis. To confirm these results, various concentrations of LTAcx were used to treat EA. After the treated cells were extensively washed they were tested for their susceptibility to lysis by complement. The same cells were also tested for the presence of cell-bound LTA using the passive hemagglutination technique (PHA) with anti-LTA. The results shown in Figures 5 and 6 indicated that both the extent of inhibition of hemolysis and PHA titers were LTAcx dose dependent. There was a decline in both activities only after the LTAcx had been diluted to a concentration of 62.5 µg/ml. The decrease in titer below this concentration indicated that the test cells were no longer saturated with LTA. There was a concomitant drop in inhibition of lysis at 62.5 µg/ml. EA which were treated with uninoculated culture medium (dialyzed Todd-Hewitt broth) were unaffected when complemnt was added.

Effect of LTAcx on lysis of sheep E and sheep E cellular intermediates. The treatment of EA with LTAcx caused the cells to become relatively resistant to complement mediated lysis. This could have been due to an effect on the antibody molecules, an effect on one or more of the complement components, or an alteration of the cell membrane.

To further investigate the nature of the complement inhibition associated with LTAcx, sheep E, sheep EA, and various sheep E complement

Figure 5. Inhibition of complement mediated lysis of EA treated with varying concentrations of LTAex.

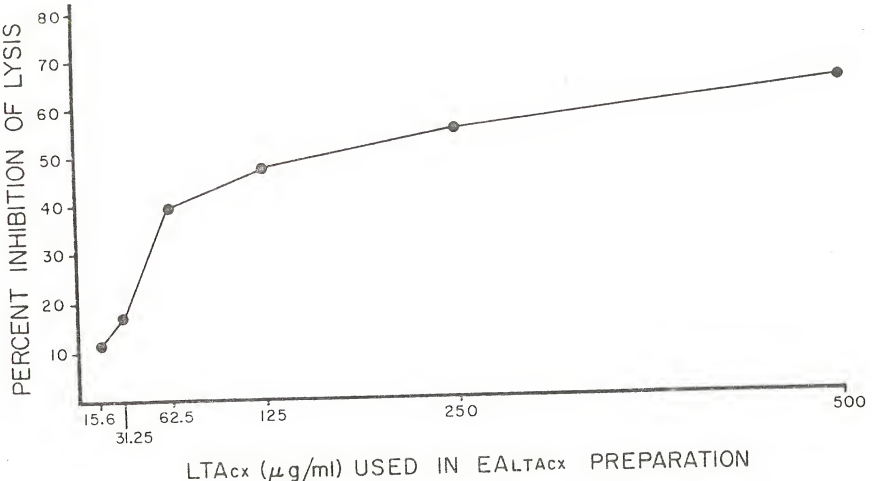
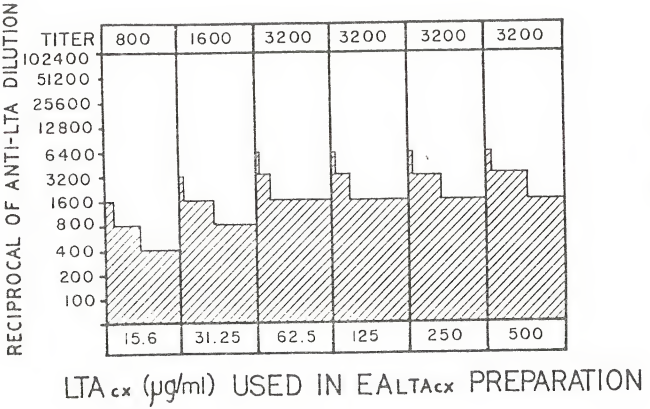

PERCENT INHIBITION OF LYSIS
80
70
60
50
40
30
20
10
15.6
31.25
62.5
125
250
500
LTAcx (μg/ml) USED IN EALTAcx PREPARATION

Figure 6. Passive hemagglutination (PHA) of EA treated with varying concentrations of LTAex.


RECIPROCAL OF ANTI-LTA DILUTION
TITER
800
1600
3200
3200
3200
3200
102400
51200
25600
12800
6400
3200
1600
800
400
200
100
15.6
31.25
62.5
125
250
500
LTA cx (µg/ml) USED IN EA LTAcx PREPARATION
0 % PHA
~50 % PHA
100 % PHA

component intermediates were treated with LTAcx and analyzed for susceptibility to complement mediated lysis. The LTAcx treated cells were also tested for bound LTA using PHA with anti-LTA. Results indicated that E, EA, and EAC$\overline{14}$ were all refractory to complement mediated lysis and that LTA was detectable on the surfaces of the cells (Figures 7 and 8). However, EAC$\overline{1423567}$ which had been treated with LTAcx were not resistant to lysis despite the fact that LTA was detectable on the cells (Figure 8). Thus, the inhibitor appeared to affect a complement component required for lysis os EAC$\overline{14}$, but which was unnecessary for lysis of EAC$\overline{1423567}$.

In an attempt of focus on the site of inhibition, the ability of LTAcx to affect the hemolytic susceptibility of EAC$\overline{142}$ was examined. This intermediate possesses C3 convertase activity (C$\overline{42}$) which is involved in the generation of SAC$\overline{1423}$ and SAC$\overline{14235}$. However, $\overline{C1}$ is not required for lysis of the intermediate once SAC$\overline{142}$ have been formed (148). Failure of LTAcx to inhibit this intermediate would indicate that C3 convertase was not the step in the complement sequence affected by the LTAcx.

Sheep EAC$\overline{142}$ were treated with LTAcx according to the protocol that has been described. For this experiment, three different amounts of C2 were used to generate EAC$\overline{142}$ from EAC$\overline{14}$. The results clearly indicated that there was no inhibition of the intermediate complex EAC$\overline{142}$ (Figure 9). Testing by PHA with antibodies specific for LTA confirmed the presence of LTA on the surfaces of the cells at the same relative concentrations found when the other intermediate complexes were tested.

Effect of LTAcx on anti-sheep erythrocyte antibodies. Some

Figure 7. Effect of LTAcx treatment on the lysis of various complement component intermediates. Each cellular intermidiate was prepared and then treated with LTAcx (125 µg/ml). Lysis was developed using procedures described in Materials and Methods. Percent inhibition was calculated by comparison against buffer treated controls.

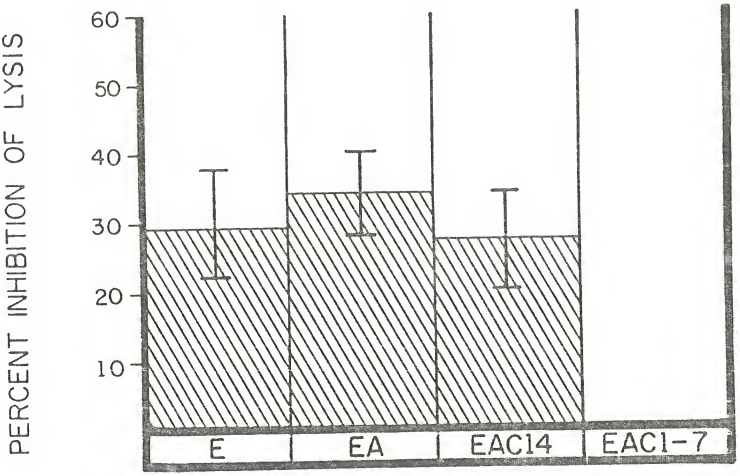
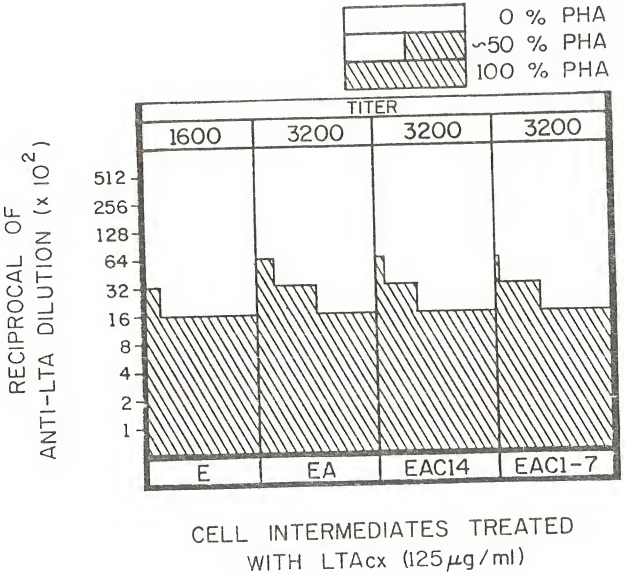

PERCENT INHIBITION OF LYSIS
60
50
40
30
20
10
E
EA
EAC14
EAC1-7
CELLULAR INTERMEDIATES TREATED
WITH LTAcx (125 μg/ml)

Figure 8. PHA of various LTAcx treated complement component intermediates.

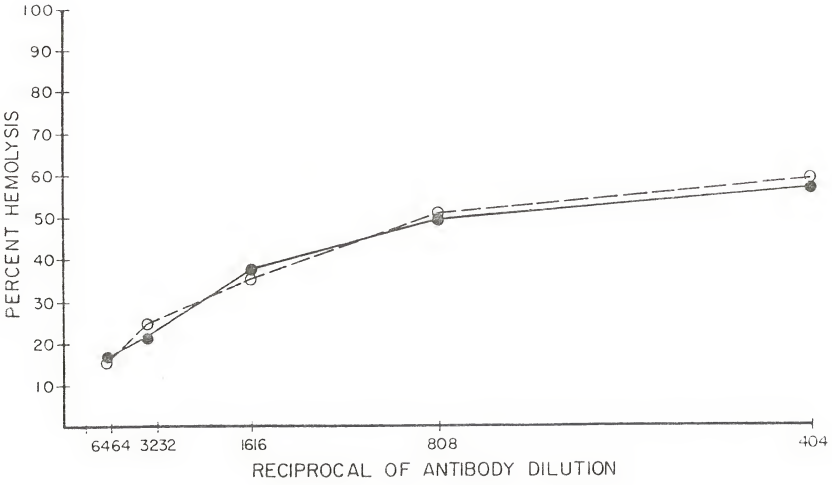

0 % PHA
~50 % PHA
100 % PHA
TITER
1600
3200
3200
3200
512
256
128
64
32
16
8
4
2
1
RECIPROCAL OF
ANTI-LTA DILUTION (x 10²)
E
EA
EAC14
EAC1-7
CELL INTERMEDIATES TREATED
WITH LTAcx (125μg/ml)

substances in LTAcx might be capable of interacting with the antibodies used to sensitize sheep E. This interaction could then lead to an impairment of $C\bar{l}$ activation and result in reduced lysis. Such a mechanism might be the reason why E and EA become resistant to lysis after treatment with LTAcx. Therefore, antibodies to sheep erythrocyte stromata were incubated with LTAcx. The mixture was then diluted to the point where the LTAcx-related inhibition could not be detected and the antibodies in the mixture were titrated (135). It was found that antibodies that had been preincubated with LTAcx had the same titer as antibodies that were incubated for the same time and temperature with VBS (Figure 10).

Partial purification of LTA. Partial purification of LTA and the complement inhibitor was accomplished by gel filtration of the LTAcx through an A-5M Biogel column. The results of a typical experiment are shown in Figure 11. Areas of antigenicity were resolved by immunodiffusion in an agarose gel utilizing an anti-serum specific for the LTA backbone. Fractions were pooled as indicated (A-F), and each pool was dialyzed against water and subsequently lyophilized. Note that pools B, C, and E contained high levels of phosphorus and that the zones of antigenicity were also located in these areas. Utilizing extracellular extracts from S. mutans and other microorganisms, similar fractionation profiles under comparable conditions were obtained by Wicken and Knox (110) and Blewiels and Craig.[1] Analysis by these workers revealed that the second phosphorous containing peak (peak II) contained LTA whereas the trailing phosphorus peak contained deacylated LTA and wall teichoic

[1] Personal communication.

Figure 10. Effect of LTAex on hemolytic antibody titration. Antibodies to sheep red blood cell stromata (Ab) were incubated with LTAex (500 µg/ml) and residual hemolysin activity was titrated by procedures described in Materials and Methods. Lysis of cells was developed with whole guinea pig complement. Symbols: (o) Ab incubated with LTAex; (●) Ab incubated with buffer.


100
90
80
70
60
50
40
30
20
10
PERCENT HEMOLYSIS
6464 3232
1616
808
404
RECIPROCAL OF ANTIBODY DILUTION

acids. Because peak II represented partially purified extracellular lipoteichoic acid, the recovered material was designated LTAppx.

A sample of each pool was rehydrated to 50 µg/ml and reacted with EA, according to standard procedures (Materials and Methods). Each EA preparation was analyzed using the complement inhibition assay and tested for bound LTA by PHA. Only the pools containing LTA (as demonstrated by PHA) caused inhibition of complement mediated lysis (Table I).

Despite the excellent separation of LTA from most of the material that absorbed light at a wave length of 260 nm, and presumably from all deacylated LTA or TA, two persistent problems arose with this purification procedure:

1). Polysaccharide contamination accounted for a major portion of the mass recovered in peak II, and

2). The total mass of LTAppx under peak II was almost immeasurably small.

In an attempt to at least increase the yield of peak II material, a Millipore Cassette system was employed to both concentrate and fractionate the spent culture supernate (Materials and Methods). This method of LTA enrichment proved highly successful as evidenced by the results in Figure 12. Even after values are corrected for the greater mass of crude extract applied on the latter column the mass yield of LTAppx was some fifteen fold greater than that obtained with previously employed procedures (Figure 11).

An analysis of results tracing the partial purification of LTA is summarized in Tables 2 and 3. It should be noted that the total amount of Pi, mass, protein, and A_{260} absorbing material decreased several thousand fold in the purification process, whereas the total

TABLE 1

Partial Purification of LTA by A5-M Gel Filtration

Pooled Fraction	Test Tube Numbers Pooled	Probable Content	Percent Inhibition of Lysis[a]	PHA Titer[b]
A	32-40	Void Volume Material	ND[c]	ND
B	41-46	LTA Plus Low Percent Carbohydrate	47.4	6400
C	47-60	LTA Plus High Percent Carbohydrate	39.1	6400
D	61-71	Carbohydrate	0.0	<100
E	72-84	TA, Carbohydrate and Nucleic Acid	0.0	<100
F	85-100	Nucleic Acid	0.0	<100
CX	-	All of Above	31.2	3200

[a] EA_{LTA} were prepared with the LTA source at a concentration of 50 ug/ml. Hemolysis was developed by incubation of the cells with several solutions of human C (37°/60 minutes). Values represent inhibition of CH_{50} units.

[b] PHA titers are expressed as the reciprocal of the final dilution of anti-LTA which still resulted in hemagglutination when incubated with the EA_{LTA}

[c] Not determined.

Figure 11. Partial purification of LTA by A5-M gel filtration. Symbols: (●) A_{260} absorbance (maximal absorbance wavelength for nucleic acids); (*) A_{485} absorbance (maximal absorbance wavelength for carbohydrates as determined by the Phenol Sulfuric Acid assay); (▲) Pi concentration in n-moles as determined by the Lowry Pi assay; (+) Antigenicity as determined by Ouchterlony gel diffusion using an antisera directed against LTA backbone.

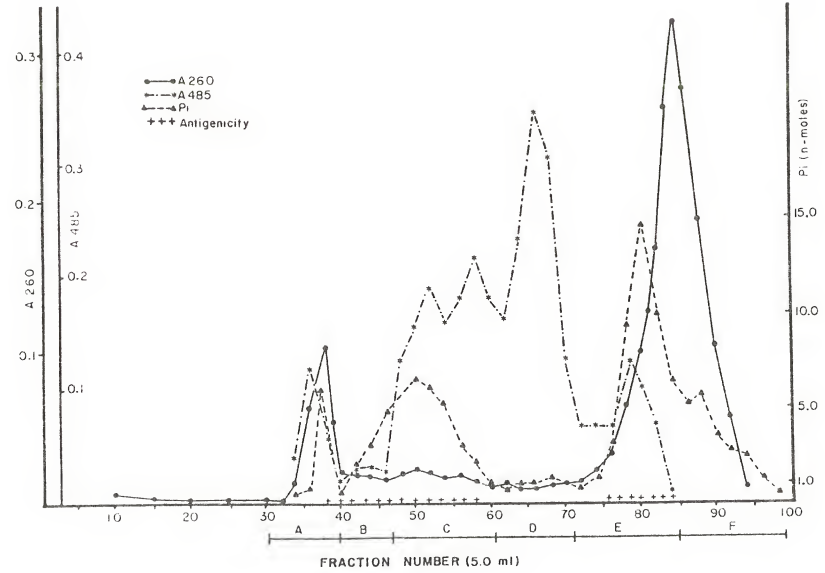

A 260
A 485
Pi
+++ Antigenicity
0.3
0.2
0.1
0.4
0.3
0.2
0.1
A 260
A 485
Pi (n-moles)
15.0
10.0
5.0
1.0
10
20
30
40
50
60
70
80
90
100
A
B
C
D
E
F
FRACTION NUMBER (5.0 ml)

Figure 12. Partial purification of LTA by A5-M gel filtration with LTA enriched starting material. Symbols: (●) A_{260} absorbance: (o) Pi concentration in u-moles/ml as determined by the Lowry Pi assay: (+) Antigenicity as determined by PHA using antisera directed against LTA backbone.

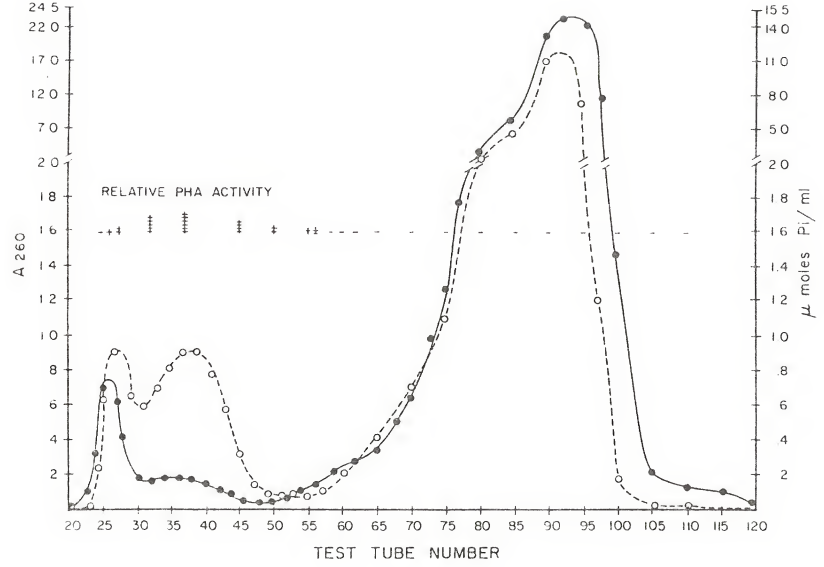

RELATIVE PHA ACTIVITY
A 260
μ moles Pi/ ml
TEST TUBE NUMBER

TABLE 2

I. Results from Partial Purification of LTA

Sample	Pi (μ-moles)[a]	Percent Lytic[b] Inhibition by LTA Treated EA	PHA Titer[c]
Dialyzed, Non-Inoculated Todd-Hewitt Broth	1.1×10^5	0	0
Supernate from Inoculated but Non-Fractionated Broth	1.0×10^5	ND	ND
PTGC Retentate Fraction of Supernate (LTAcx)	6.9×10^2	48	3200
Peak II from A5M After Desalting (LTAppx)	6.0×10^1	55	3200

[a] Data are expressed in the units indicated and represent values extrapolated back to the undilute sample times total volume.

[b] EA_{LTA} were prepared with the LTA source at a concentration of 250μg/ml. Hemolysis was developed as described in Table 1.

[c] PHA titers were determined by methods described in Table 1.

Figure 9. Effect of LTAcx treatment on the lysis of $\overline{EAC142}$. Various limiting concentrations of C2 were used to prepare $\overline{EAC142}$ cellular intermediates. The cells were then treated with LTAcx (250 µg/ml) and lysis was developed using procedures described in Materials and Methods. Symbols: (o) $\overline{EAC142}$ incubated with LTAcx; (•) $\overline{EAC142}$ incubated with buffer.

TABLE 3

II. Results from Partial Purification of LTA[a]

Sample	A260	Protein[b] (mg)	Amount of LTA in[c] Sample (mg)	Weight of Sample (mg)
Dialyzed, Non-Inoculated Todd-Hewitt Broth	3.7×10^5	6.5×10^3	0.0	3.3×10^5
Supernate from Inoculated but Non-Fractionated Broth	3.6×10^5	6.7×10^3	9.1	ND
PTGC Retentate Fraction of Supernate (LTAcx)	1.25×10^3	9.5×10^2	11.0	2.3×10^3
Peak II from A5M After Desalting (LTAppx)	1.76×10^1	6.0	8.2	3.6×10^1

[a] Unless otherwise indicated, all data are expressed in the units indicated and represent values extrapolated back to the undilute sample times total volume.

[b] Values determined by Bio Rad Protein Assay.

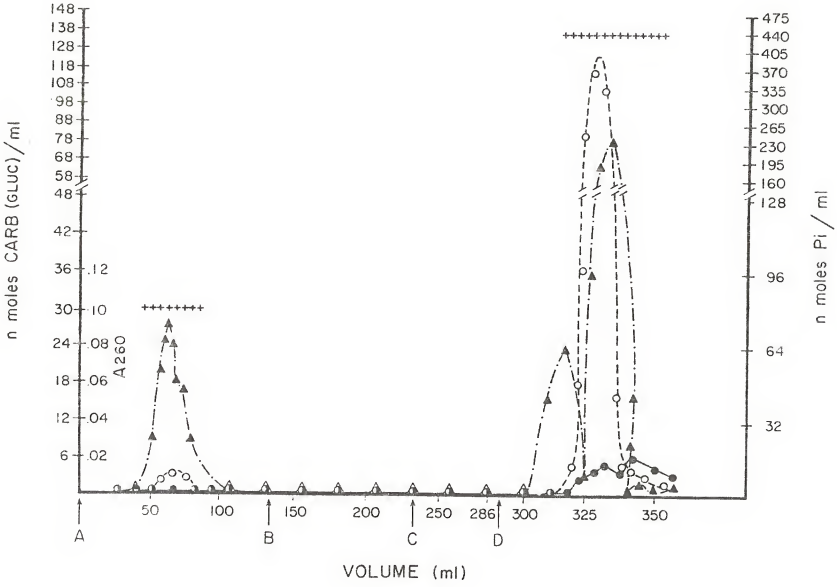
[c] These values were calculated by determining the minimal concentration of purified LTA that can still be detected by PHAg. Equating this value with the PHAg end point for all other LTA sources, the hypothetical LTA concentration in the starting well can be calculated by serial twofold interpolations.

amount of LTA in the sample, PHA titer, and percent lytic inhibition of EA remained relatively unchanged or increased in value.

Purification of LTA by hydrophobic interaction gel chromatography. A 25.0 X 2.25 cm column packed with Octyl Sepharose and equilibrated in buffer A was prepared as described in Materials and Methods. Approximately 6.0 mg of LTAppx dissolved in 10.0 ml of buffer A were applied to the column. As can be seen in Figure 13, a small amount of phosphate containing material passed unimpeded through the column. A slightly greater mass of polysaccharide was also excluded without binding. No additional material eluted from the column with buffer B. Point C on the graph marks the location where a 10-70% propanol gradient was begun. Point D represents the point where a significant volume decrease per test tube was observed. Since fractions were collected on a "drops per tube" basis, the presence of propanol in the effluent causes a change in surface tension of the drop resulting in a decreased volume per drop. The ultimate result is a decrease in the volume per tube. This, test tube volume provided a convenient means of monitoring the progress of the propanol gradient.

It should be noted that despite the use of a gradient (the original procedure called for a single step-wise elution with 50% propanol) significant amounts of carbohydrate eluted with the LTA. As indicated on the the graph, all areas containing phosphates also contained LTA as detected using PHA. The fact that a small amount of LTA passed unbound through the column suggests that either the columns binding capacity was exceeded, or perhaps the LTA was only partially acylated and not capable of tenacious hydrophobic binding.

Figure 13. Purification of LTA by Octyl Sepharose hydrophobic gel chromatography. Symbols: (●) A_{260} absorbance; (▲) concentration of carbohydrate (n-moles/ml) as determined by the Phenol-Sulfuric Acid assay. Concentrations were determined using glucose as a standard carbohydrate. (o) Concentration of Pi (n-moles/ml) as determined by the Lowry Pi assay; (+) zones of antigenicity as determined by PHA using antisera directed against LTA backbone; (A) elution with buffer A (1.0M NaCl, 0.01 M Tris-carbonate pH 6.8); (B) elution with buffer B (0.01 M Tris-carbonate, pH 6.8); (C) elution with a 10-70° gradient of a propanol-buffer B mixture; (D) elution volume at which significant reductions of volume/tube were observed, indicating elution of propanol.


n moles CARB (GLUC) / ml
148
138
128
118
108
98
88
78
68
58
48
42
36
30
24
18
12
6
A260
.12
.10
.08
.06
.04
.02
n moles Pi / ml
475
440
405
370
335
300
265
230
195
160
128
96
64
32
50
100
150
200
250
286
300
325
350
A
B
C
D
VOLUME (ml)

All test tubes containing greater than 25.0 n-moles Pi/ml were pooled. The entire peak (approximately 22 ml) was loaded on to a 65.0 cm X 3.0 cm column packed with LH-20 equilibrated with deionized water. Four and two tenths milliliter of effluent were collected per test tube a a flow rate of approximately 30.0 ml/hour. The results of this procedure, which simultaneously removed salt and propanol, are shown in Figure 14. The column effluent was monitored at a wave length of 220 nm and was also screened for LTA by PHA (++++) using a single dilution sample. In addition column fractions were tested for the presence of chloride ions by placing one drop of a saturated $AgNO_3$ solution on a coverslip containing one drop from each test tube. Any resulting precipitation was evaluated on a +1 to +5 basis and plotted accordingly. It was empirically determined that not only Cl reacted with the $AgNO_3$ resulting in insoluble AgCl, but the NaN_3 and tris carbonate in the buffers reacted as well. The presence of propanol was monitored indirectly by changes in test tube volume. Since LTA, azide, and tris carbonate all absorb at a wave length of 220 nm the combination of ultraviolet light screening, the $AgNO_3$ precipitation test, and visual inspection of volume changes per test tube proved to be invaluable for rapidly discerning the location and separation of LTA from contaminating salts and solvents. The entire contents of peak I were pooled, frozen, and lyophilized. The final product was referred to as LTAosx (extracellular lipoteichoic acid purified by Octyl Sepharose hydrophobic affinity gel chromatography). The typical mass yield from such a procedure was about 60-70%. Percent recovery of LTA at various points in the procedure is summarized in Table 4.

Figure 14. Simultaneous removal of salt and propanol free LTAosx by LH-20 gel chromatography. Symbols: (•) A_{220} absorbance; (o) Volume/test tube: (+) Antigenicity as determined by PHA; (Shaded Area) relative degree of precipitation of salt and other low molecular weight materials as determined by $AgNO_3$ test.

A 220
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
5
10
15
20
25
30
35
40
45
50
TEST TUBE NUMBER
AgNO3 TEST
+5
+4
+3
+2
+1
5.0
4.0
3.0
2.0
1.0
VOLUME / TEST TUBE

TABLE 4

Percent Recovery[a] of LTA During Octyl Sepharose Purification

LTA Source	Reciprocal of Initial Dilution	Initial Concentration of LTA (µg/ml)[b]	Total volume of Sample (ml)	Calculated Total Weight of LTA in Sample (mg)	Percent Recovery of LTA
LTAppx	400	0.500	9.0	1.80	100.0
Peak I	2	0.250	87.0	0.04	2.2
Pooled column Effluent from all areas not Located Under Peak I or II	2	0.000	273.4	0.00	0.0
Peak II (LTAosx) Before Passage through LH20	100	0.500	31.6	1.58	87.8
Peak II (LTAosx) After Passage through LH20	100	0.25	62.3	1.56	86.5

[a] As determined by PHAg

[b] Determined by methods described in Table 3

[c] Calculated by multiplying the corresponding values for the first three columns

Phosphatidyl choline vesicle (PVC) purification of LTA using ^{14}C labelled phosphatidyl choline. Approximately 5 X 10^6 DPM of ^{14}C labelled phosphatidyl choline were added to 40 mg of phosphatidyl choline dipalmitoyl. Phosphatidyl choline vesicles (PVC) were prepared as described in Materials and Methods. Three test tubes containing identical volumes and concentrations of non-labelled PCV were prepared simultaneously and 2.0 ml of LTAppx (1.5 mg/ml) were added to each test tube. Fifty microliter samples from the ^{14}C containing test tube were removed at various steps during the purification process and analyzed as described (Materials and Methods). A standard chloroform quench curve was constructed and all reported counts represent corrected DPM values. Table 5 depicts the distribution of ^{14}C counts at various steps in the purification procedure. Utilizing this procedure as described, essentially no contaminating phospholipid could be detected in the final product. The typical mass yield of product via PVC purification was about 10-15%. Percent recovery of LTA at various steps in the procedure is summarized in Table 6.

Comparison and summary of LTApcx versus LTAosx. As indicated in Table 7, both methods of LTA purification removed the majority of protein as compared to the total amount available in the LTAppx. Both methods ostensibly recovered > 80% of the original LTA. However, the major difference between the two products is reflected in the percent total mass recovery and the concomitant increase in percent carbohydrate in the final LTAosx product. This latter difference can be most readily discerned by observing the composite gas chromatograph tracings in Figure 15. The carbohydrate standard (CHO-STD) depicts the typical chromatograph of glucose and galactose after preparing trimethylsilyl

Distribution of ^{14}C-Phosphatidyl Choline During PCV Purification of LTA

Sample Source	Reciprocal of[a] Dilution Factor	DPM Aliquot[b] ($\times 10^{2}$)	Total DPM in Sample ($\times 10^{3}$)	Percent of Total DPM	Calculated Corresponding Weight of PCV (mg)
^{14}C-PCV Suspended in Starting Buffer	60	830.00	4980.00	100.00	40.00
Supernate from Preliminary Vesicle Washing (#1)	320	1.91	61.12	1.23	0.49
Supernate from Preliminary Vesicle Washing (#2)	320	0.29	9.28	0.19	0.07
Decanting After Reaction of PCV with LTAppx	320	2.95	94.40	1.90	0.76
1st Washing Supernate	320	2.82	90.24	1.81	0.72
2nd Washing Supernate	320	1.61	51.52	1.03	0.41
3rd Washing Supernate	320	3.44	110.08	2.21	0.88
Chloroform/Methanol Filtrate	72	590.00	4248.00	85.30	34.12
1st Chloroform/Methanol Washing	100	35.00	350.00	7.03	2.81
1st Chloroform Only Washing	60	1.09	6.54	0.13	0.05
2nd Chloroform Only Washing	60	0.58	3.45	0.07	0.03
Final Product (LTApcx)	1[c]	1.96	0.20	0.00	0.00

[a] Dilution factor was calculated by dividing the total volume of the sample by the volume of the aliquot removed for analysis.

[b] DPM values were calculated from CPMs and a standard quench curve as described in Materials and Methods.

[c] ^{14}C contamination of the final product was determined by placing the entire LTA-containing floropore filter in a scintillation vial and analyzing as described in Materials and Methods.

TABLE 6

Percent Recovery[a] of LTA from Various Steps of PCV Purification

LTA Source	Reciprocal of Initial Dilution	Initial Concentration[b] of LTA (µg/ml)	Total Volume of Sample (ml)	Calculated Total[c] Weight of LTA in Sample (mg)	Percent Recovery of LTA
LTAppx	600	0.500	6.0	1.80	100.0
Decant	5	0.125	48.0	0.03	1.6
1st Wash	1	0.000	48.0	0.00	0.0
2nd Wash	1	0.000	48.0	0.00	0.0
3rd Wash	1	0.000	48.0	0.00	0.0
LTApcx	1000	1.000	1.5	1.50	83.3

[a] As determined by PHAg

[b] Determined by methods described in Table 3

[c] Calculated by multiplying the corresponding values of the first 3 columns

TABLE 7

Summarized Chemical Composition of Various LTA Containing Sources

LTA Source	Percent Composition of Dry Weight							
	Carbohydrate[a]	Protein		LTA[b]	Pi[c]	A_{220}[f]	A_{260}	A_{280}
		Amino Acid Analysis	Bio-Rad Protein Assay					
LTA ppx	23-32	21-28	16-22	21-26	1.8-2.5	.555	.090	.082
Combined Fractions from Octyl Sepharose (except Peak II)	57-65	43-56	36-48	<5	0.9-1.3	.392	.105	.071
LTAosx (Peak II, Octyl Sepharose)	15-30	-	4-6	72-85	3.1-4.0	.368	.091	.074
Combined Supernates from PCV washings	49-58[d]	26-34[d]	16-22[d]	<5	NA[e]	NA	NA	NA
LTApcx	<5	-	<2.5	<95	5.8-6.2	.160	.093	.069

[a] Percent carbohydrate was determined by gas liquid chromatography as described in Materials and Methods.

[b] Percent LTA was determined by PHAg.

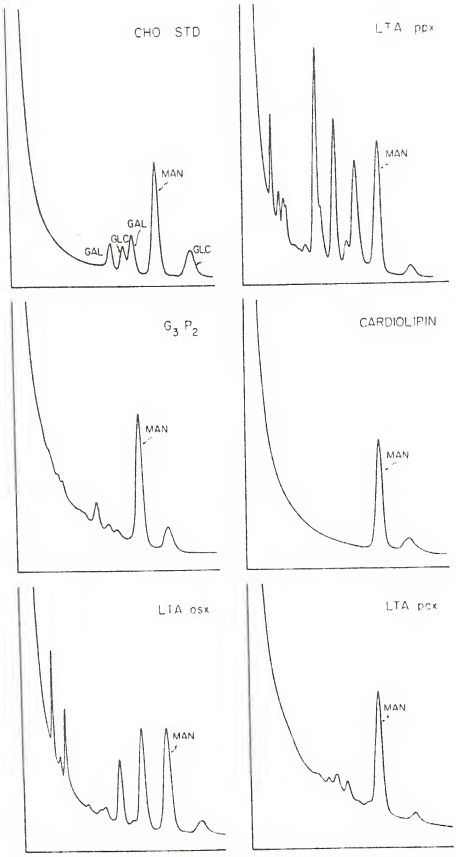
[c] Percent phosphate was determined by the Lowry Phosphate assay.

[d] These values were corrected for weight differences due to contaminating phospholipid vesicles.

[e] Because of the high percent phospholipid vesicle contamination in this sample, valid determinations for total Pi and optical densities were not possible.

[f] Ultra violet light absorbance determinants were made with the indicated materials at a concentration of 100 µl/ml in distilled water.

Figure 15. Carbohydrate analysis of LTA containing preparations by gas liquid chromatography. Abbreviations: (MAN) Mannitol; (GLC) Glucose; (GAL) Galactose. Mannitol was incorporated as an internal standard with all samples.


CHO STD
GAL
GLC
GAL
MAN
GLC
LTA ppx
MAN
G_3P_2
MAN
CARDIOLIPIN
MAN
LTA osx
MAN
LTA pcx
MAN

ester (TMS) derivatives as described in Material and Methods. An internal mannitol standard is included with all samples. The LTAppx chromatograph represents the typical carbohydrate profile achieved with partially purified LTA. The tracings for LTAosx and LTAppx contrast the qualitative and quantitative differences in carbohydrate content. The second two chromatograms, deacylated cardiolipin (G_3P_2)[1] and cardiolipin, were included as a comparison of how a naked polyglycerol phosphate backbone might be expected to react under the described conditions. The base line instability of the G_3P_2 looks remarkably similar to the profile of the purified LTApcx. The procedure for purifying deacylated cardiolipin requires passage through Sephadex columns. It is quite conceivable that the minute quantities of unidentified carbohydrates which are indicated may be due to dextran contamination from the column. However, it would be difficult to account for the same source of contamination for the LTApcx since gel chromatography was not used in the final purification. On the other hand, the similarity of the indicated chromatogram tracings may be more than mere coincidence and may reflect actual reactions of the derivatizing agent with the polyglycerol phosphate backbone. This latter hypothesis is supported by the fact that an unidentified trailing "carbohydrate" peak of significant mass appears in both the cardiolipin and G_3P_2 chromatographs. The Rf value of this peak is similar (but yet suspiciously disparate) to the retention time normally observed for β-glucose. However, if indeed this peak does represent β-glucose, one is hard pressed to rationalize why a corresponding α-glucose peak does not occur as well. In either case, it is

[1] Deacylated cardiolipin was prepared by the method of Wilkinson (149) and was kindly provided by R. Craig, University of Florida.

apparent that the LTAosx still contains significant amounts of carbohydrate contamination in contrast to the lower yield, but highly purified LTApcx.

A summary of specific activities relative to PHA activity is presented in Table 3.

Inhibition of complement mediated lysis of LTApcx treated EA. Once a highly purified preparation of LTA was obtained, it was necessary to confirm the results that had been previously established with LTAcx. As can be seen in Figure 16, not only were LTApcx treated EA refractory to complement mediated lysis, but in addition the general profile was remarkably similar to LTAcx treated EA. As indicated, LTApcx was used in concentrations five-fold to ten-fold less than those used with LTAcx to achieve comparable degrees of inhibition. PHA titers typically indicated LTA saturation of the cells.

Effect of LTApcx on the lysis of various cellular complement component intermediates. Sheep E, EA , and various complement intermediates were treated with LTApcx (100 ug/ml in DVB) and washed extensively in DGVB. Percent lysis and inhibition of CH_{50} units were determined as described in Materials and Methods. The results of these experiments are summarized in Figure 17. As with LTAcx treated cells, E, EA, and $EAC\bar{1}$ were all refractory to lysis by complement. Likewise, $EAC\bar{1}\bar{4}\bar{2}$ and $EAC\bar{1}-\bar{7}$ intermediates were totally unaffected by the presence of LTApcx on their cell surfaces. The only observable difference in the activity of LTApcx on cellular intermediates versus LTAcx was that $EAC\bar{1}\bar{4}$ cells were inhibited to a lesser degree with LTApcx than LTAcx.

Effect of LTAcx and LTApcx on fluid phase $C\bar{1}$. As previously discussed, $C\bar{1}$ is not required for lysis once $EAC\bar{1}\bar{4}\bar{2}$ are formed but it is

TABLE 8

Comparing Several Different Methods of Measurement[a]

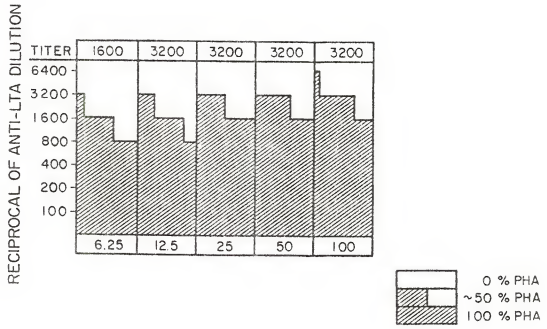
LTA Source	PHAg	Protein X 10^{-3}	Carbohydrate X 10^{-3}	A260 X 10^{-3}	Pi X 10^{-3}	Mass X 10^{-3}
LTAcx	400	(3.4)	N.D.[b]	(0.3)	(4.6)	(1.4)
LTApx	2,400	(96)	(55.7)	(26,667.0)	(1,116.0)	(1,333.3)
LTAosx	8,000	(1,600)	(347.8)	(87,912.0)	(2,222.2)	(5,128.2)
LTApcx	32,000	(12,800.0)[c]	(6.400)[c]	(344,086.0)	(5,203.2)	(21,333.3)

[a] Protein, carbohydrate, phosphorus (Pi) and mass calculations were made on the basis of activity (expressed on the reciprocal of PHAg titer) per mg.

[b] Not determined.

[c] These values are estimations since the amounts of material present were below the limits of detection for the methods.

Figure 16. Passive hemagglutination (PHA) titration and inhibition of complement mediated lysis of EA treated with varying concentrations of LTApcx.


RECIPROCAL OF ANTI-LTA DILUTION
TITER
1600
3200
3200
3200
3200
6400
3200
1600
800
400
200
100
6.25
12.5
25
50
100
0 % PHA
~50 % PHA
100 % PHA

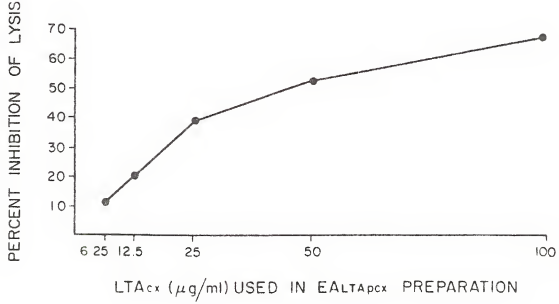
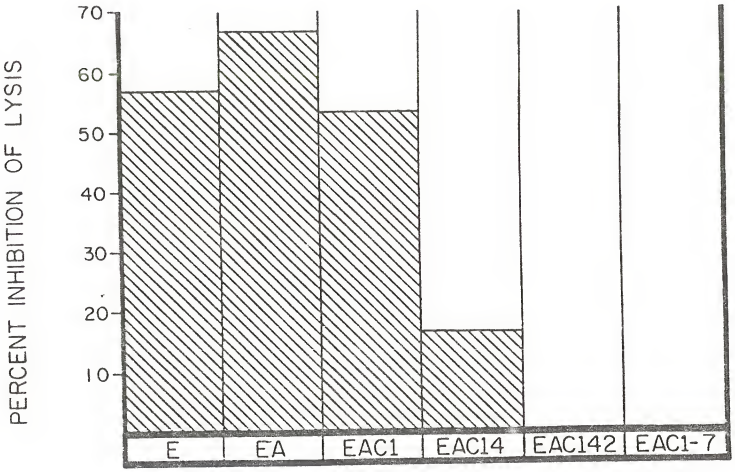

PERCENT INHIBITION OF LYSIS
70
60
50
40
30
20
10
6.25
12.5
25
50
100
LTAcx (μg/ml) USED IN EALTApcx PREPARATION

Figure 17. Effect of LTApex on the complement mediated lysis of various cellular complement component intermediates.


PERCENT INHIBITION OF LYSIS
70
60
50
40
30
20
10
E
EA
EAC1
EAC14
EAC142
EAC1-7
CELLULAR INTERMEDIATES TREATED
WITH LTApcx (100 μg / ml)

essential until that point is reached (148). In addition, many polyanionic substances are known to directly affect C1 by interfering with C1q binding or C1 esterase ($C\overline{1s}$) activity (39,40). Because LTA is polyanionic due to the polyglycerol phosphate backbone and because cellular intermediates beyond the $EAC\overline{142}$ step were no longer inhibited, it seemed reasonable to hypothesize that LTA was behaving like a polyanion and directly affecting C1.

To test this hypothesis, LTAcx and LTApcx were preincubated with functionally purified human $C\overline{1}$ at 30°/15 minutes. Residual $C\overline{1}$ activity was titrated as described by Rapp and Borsos (139) and activity was compared against buffer treated controls. The results shown in Figure 18 indicate that although LTAcx consumed $C\overline{1}$ activity, purified LTApcx did not.

Purification of human C1q, C1s and $C\overline{1s}$. In an attempt to further eludicate a possible site and mechanism of C inhibition, human C1 subcomponents were purified by the methods and modifications previously described (Materials and Methods). Although homogeneity beyond functional purity was not essential, the methods employed yielded highly purified products. Figure 19 demonstrates C1q homogeneity by immunodiffusion against several monospecific antisera. Precipitation bands of identity were observed in adjacent wells containing the whole human serum starting material, the purified C1q final product, and a highly enriched C1q prior to final precipitation (Figure 19, plate 5, well numbers A, G, and E). As can be observed in Figure 20, disc gel electrophoresis of the final product revealed a single dark staining band which barely migrated into the separation gel. These results are consistant with the observations of other investigators (144).

Figure 18. Effect of LTAcx and LTApcx on functionally purified human $\overline{C1}$. The upper graph represents a residual $\overline{C1}$ titration after incubation with LTAcx (500 ug/ml). The lower graph represents the results from an analogous experiment using LTApcx (500 ug/ml) instead of LTApcx in the incubation mixture. Symbols: (●) $\overline{C1}$ incubated with buffer; (o) $\overline{C1}$ incubated with the appropriate LTA containing extract.

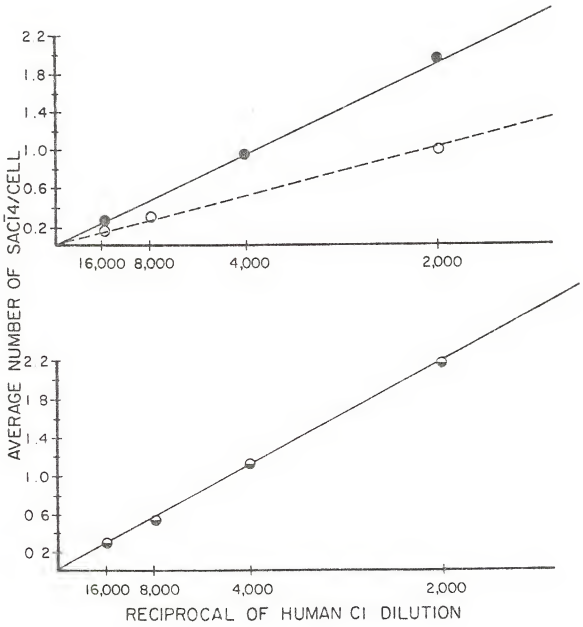

AVERAGE NUMBER OF SAC14/CELL
2.2
1.8
1.4
1.0
0.6
0.2
16,000 8,000 4,000 2,000
2.2
1.8
1.4
1.0
0.6
0.2
16,000 8,000 4,000 2,000
RECIPROCAL OF HUMAN CI DILUTION

Figure 19. Immunodiffusion and precipitation analysis of various steps in the purification of human Clq. Purification was achieved by repeated fractional precipitations of whole human sera in buffers varying in ionic strength, pH, and concentrations EGTA or EDTA (144).
Well designations:
(A) Whole human sera (starting material);
(B) Supernate from first precipitation;
(C) Supernate from second precipitation;
(D) Supernate from third precipitation;
(E) Material from resuspended pellet prior to final precipitation.
(F) Final product (purified Clq).
Plate designation: (1) Center well contains anti-IgG; (2) Center well contains Anti-IgA; (3) Center well contains anti-whole human sera: (4) Center well contains anti-IgM: (5) Center well contains anti-Clq.

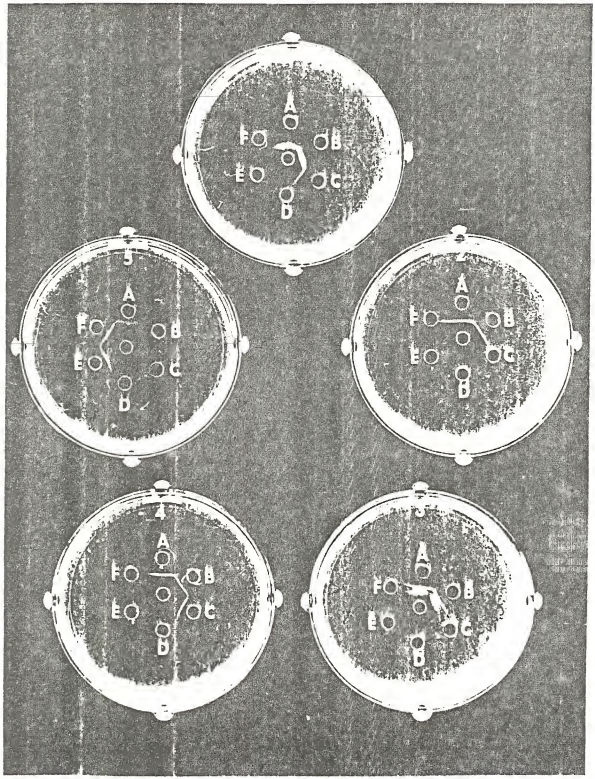
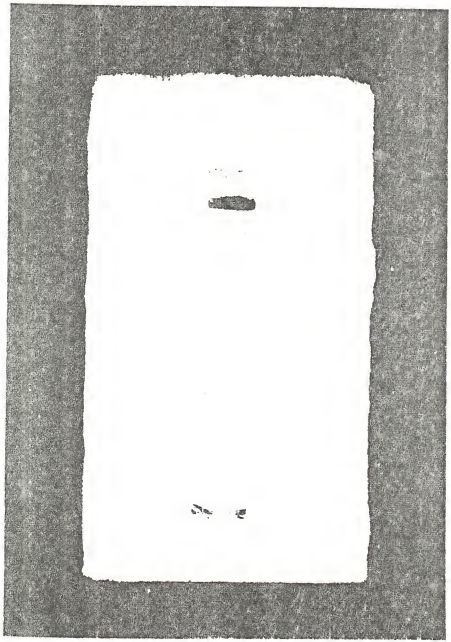

A
F
B
E
C
D
3
4
5

Figure 20. Disc gel electrophoresis of purified human C1q. Cathode was at the top.



The procedures for C1s and $\overline{C1s}$ purification were modified only in that an ionic gradient was used in the final purification step of both reagents rather than the stepwise elution utilized by Sakai and Stroud (35). The rationalization for this modification was that a difference in binding capacities of the DEAE matrix could have deleteriously effected the elution characteristics of the C1s ($\overline{C1s}$) at a fixed ionic strength. The elution profile of C1s is shown in Figure 21. It should be noted that two peaks of material which absorbed light at a wave length of 280 nm were resolved during the gradient elution. Both peak II and and peak III reacted with monospecific antisera to C1s, however, only peak III contained C1s activity. Peak II presumably represents an inactive form of either C1s or $\overline{C1s}$. No such extraneous peak was resolved during DEAE chromatography of $\overline{C1s}$.

Immunoelectrophoretic analysis of purified human C1s and $\overline{C1s}$ on 1% Noble Agar is depicted in Figure 22. Results indicate a difference in electrophoretic mobility of C1s and $\overline{C1s}$ which is consistent with the observations of previous investigators (35). Also, there was a "gull wing" pattern displayed by $\overline{C1s}$ apparently representing microheterogeneity of the activated proesterase. This too has been observed by previous investigators (35).

Effect of LTApcx on the ability of $\overline{C1s}$ to consume C4 and C2 activity. As previously discussed, activated C1 esterase ($\overline{C1s}$) is capable of cleaving C4 into C4a and C4b (43) as well as cleaving C2 into C2a and C2b (46). In either case, the active fragments rapidly decay and if not quickly attached to membrane sites, lose their ability to do so. The ephemeral nature of these active fragments can be used as sensitive indices of $\overline{C1s}$ activity. As described in Materials and Methods, equal

Figure 21. DEAE elution profile of human C1s. Peak I contains C1s activating proteins (functionally pure C1r); Peak II contains nonfunctional C1s; Peak III contains functional, non-activated C1s. Symbols: (•) Absorbance at A_{280} (maximal absorbance for most proteins; (o) Relative salt concentration (RSC) as measured by electroconductivity. Arrows indicate the addition of high ionic strength Sodium Chloride buffer.

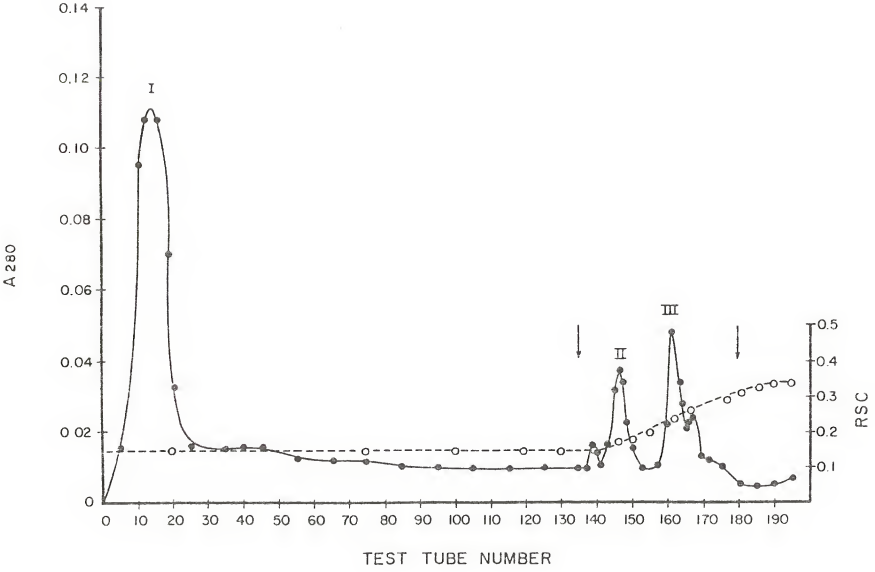
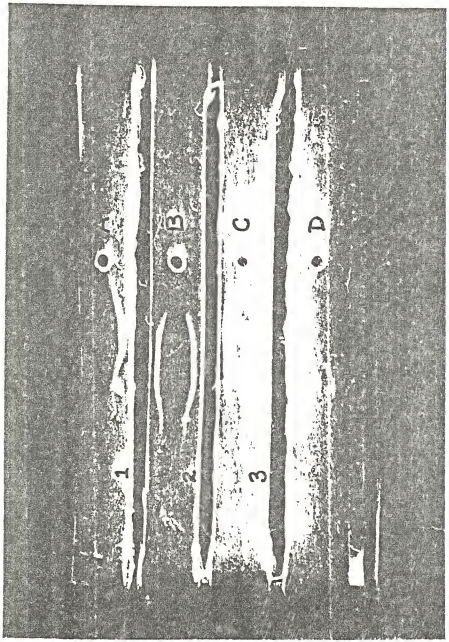

0.14
0.12
0.10
0.08
0.06
0.04
0 02
0
A 280
I
II
III
0.5
0.4
0.3
0.2
0.1
RSC
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190
TEST TUBE NUMBER

Figure 22. Immunoelectrophoresis of human C1s and $\overline{C1s}$. Well A and D contained purified C1s; well B contained purified $\overline{C1s}$; well C contained whole human sera. Trough 1 contained anti C1s ($\overline{C1s}$); troughs 2 and 3 contained a mixture of 75% anti whole human sera and 25% anti C1s ($\overline{C1s}$).


A
B
C
D
1
2
3

volumes of $\overline{C1s}$, functionally pure C4 or C2, and LTApcx were mixed and incubated at 37°/5 minutes. The incubation mixture was then serially diluted in DGVB, and the residual titers of C4 or C2 were determined and compared against a buffer treated control. As shown in Table 9, no appreciable difference in $\overline{C1s}$ activity can be observed when even 200 µg/ml of LTApcx were used. Also note that LTApcx preincubated with either C4 (Expt. Group 2d) had no significant effect on residual activity.

Effect of LTApcx on the ability of $\overline{C1s}$ to hydrolyze TAMe. The ability of $\overline{C1s}$ to hydrolyze the synthetic substrate TAMe is another index of $\overline{C1s}$ activity. As can be seen in Figure 23, essentially no inhibition of $\overline{C1s}$ activity was observed when $\overline{C1s}$ is preincubated with LTApcx and TAMe.

Effect of LTApcx on the ability of C1q to bind to target cells. Since C1q is the recognition unit of the classical pathway of complement, any alternation in its ability to react with the antigen-antibody complexes on the surface of EA would have profound affects on the ability of complement to lyse those cells. Therefore, equal volumes of purified human C1q and LTApcx (10 µg/ml) were preincubated at 30°/15 minutes. After preincubation, C1q was serially diluted in DGVB and C1r and C1s reagents were added. Hemolysis was then developed as described (Materials and Methods). Again there was no apparent inhibition (results not shown) of activity. The major criticism of this experiment is that the LTApcx concentration used to preincubate with C1q is 10-fold less than what was normally used in fluid phase inactivations. The reason for the use of this lower concentration was to insure that the LTA would be sufficiently diluted at the time of EA addition. If significant amounts of LTA were present in the incubation mixture, EA_{LTA} would form, thus generating a false positive inhibition due to the refractory nature of

TABLE 9

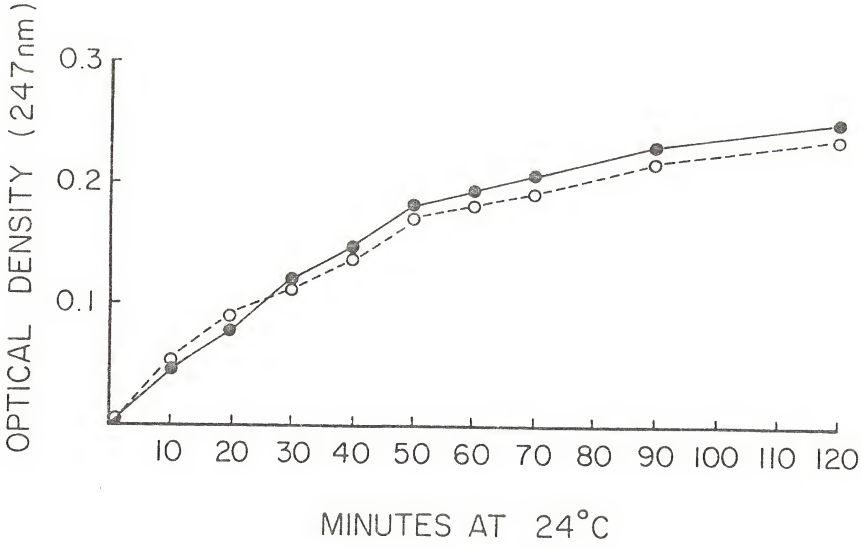
Effect of LTApcx on the Ability of $\overline{C1s}$ to consume C4 and C2 Activity

Expt. Group	DGVB	$\overline{C1s}$	C4	C2	LTApcx (50 µg/ml)	LTApcx 200 µg/ml)	Residual C4 or C2 Activity	Consumption of C4 or C2 Activity
	µl	µl	µl	µl	µl	µl	SFU/ml[a]	%
1a	100	100	100				6.2×10^9	86
b		100	100		100		7.1×10^9	84
c		100	100			100	8.6×10^9	81
d	100		100			100	4.3×10^{10}	4
e	200		100				4.5×10^{10}	NA[b]
2a	100	100		100			6.5×10^9	93
b		100		100	100		9.1×10^9	90
c		100		100		100	1.2×10^{10}	87
d	100			100		100	9.4×10^{10}	0
e	200			100			9.2×10^{10}	NA

[a] Site forming unit.

[b] Not applicable

Figure 23. Effect of LTApex on the ability of Cls to hydrolize TAMe. As the synthetic substrate TAMe is hydrolized, there is an increase in A_{247} absorbing material. In this experiment, Cls and TAMe were incubated together in the presence of LTApex (100 µg/ml) at room temperature (24°C). Symbols: (●) Cls and TAMe plus buffer; (○) Cls and TAMe plus LTApex.


OPTICAL DENSITY (247nm)
0.3
0.2
0.1
10 20 30 40 50 60 70 80 90 100 110 120
MINUTES AT 24°C

EA_{LTA}. Despite the lower concentration, previous data with LTA treated EA (Figure 16) indicated that even at this concentration, inhibition should have been significant if indeed Clq were the site of inhibition. Instead of this anticipated inhibition, 1.36×10^8 SFU/ml of Clq were recovered from the incubation mixture originally containing 1.50×10^8 SFU Clq/ml. Due to assay variation this difference was considered insignificant.

Effect of LTApcx on Cl transfer. In another attempt to elucidate the effect of LTA on the Cl molecule, the interference of the normal ability of Cl to transfer from cell to cell under conditions of high ionicity was investigated. Two different types of transfer tests were performed. In type I, EA were treated with LTA and then $C\bar{1}$ was added. In type II, $EAC\bar{1}$ were prepared and then LTA was added. Not so surprisingly there was no inhibition of Cl transfer as measured by hemolysis of EAC4 cells. However, there was an increase in the Cl transferability of cells containing LTA. As can be seen in Table 10, this phenomenon was repeatable and was observed in both types of experiments.

Differences in complement mediated lytic susceptability of LTAcx treated $EAC\bar{4}$ versus $EAC\bar{1}\bar{4}$. Buffer or LTAcx (250 µg/ml) was used to treat $EAC\bar{4}$ using procedures described in Materials and Methods. Various limiting concentrations of human $C\bar{1}$ were then added to aliquots of the cells and lysis was developed as previously described. Alternately, $EAC\bar{1}\bar{4}$ were prepared using various limiting concentrations of human $C\bar{1}$. Aliquots of cells were then treated with LTAcx (250 µg/ml) or with buffer. After the cells were washed extensively in buffer, lysis was developed as previously described. As shown in Figure 24, $EAC\bar{1}\bar{4}$ treated with LTA are considerably more refractory to complement mediated lysis than are $EAC\bar{4}_{LTA}$ treated with $C\bar{1}$.

TABLE 10

Comparison of the Relative Numbers of Effective $\overline{Cl}$ Molecules Capable of Transfer from EA$\overline{Cl}$ Treated with LTApcx

Sample	Experiment Number	Effective Number of $\overline{Cl}$ Molecules Transferred/Cell
EA$\overline{Cl}_{LTA}$ [a]	1	175
EA$\overline{Cl}_{LTA}$	2	124
EA$\overline{Cl}_{LTA}$	3	153
EA$\overline{Cl}_{DVB}$ [b]	1	137
EA$\overline{Cl}_{DVB}$	2	115
$EA_{LTA}\overline{Cl}$ [c]	1	185
$EA_{LTA}\overline{Cl}$	2	178
$EA_{LTA}\overline{Cl}$	3	215
$EA_{DVB}\overline{Cl}$ [d]	1	132
$EA_{DVB}\overline{Cl}$	2	182

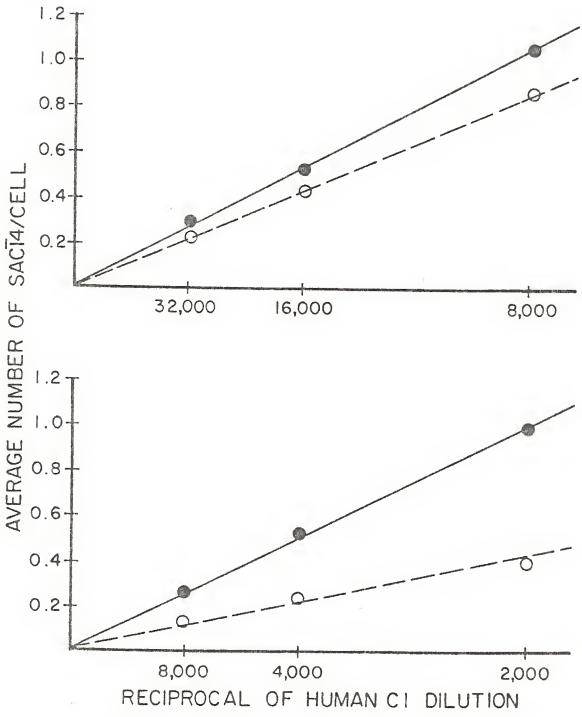
[a] EA$\overline{Cl}$ were generated and treated with LTApcx at 100 µg/ml in DVB. After extensive washing, the $\overline{Cl}$ capable of transfer was titrated.

[b] Control EA$\overline{Cl}$ treated with DVB for 30°/15 minutes

[c] EA_{LTA} were prepared (100 µg LTApcx/ml) and after the cells were extensively washed $EA_{LTA}\overline{Cl}$ were generated. The $\overline{Cl}$ capable of transfer was then titrated.

[d] Control EA$\overline{Cl}$ treated with DVB while in the FA state. EA$\overline{Cl}$ preparation and $\overline{Cl}$ transfer exactly paralleled the LTA treated cells.

Figure 24. Differences in complement mediated lytic susceptability of LTAcx treated $EAC\overline{4}$ versus $EAC\overline{14}$. Upper graph: $EAC\overline{4}$ were treated with buffer or with LTAcx (250 µg/ml). Various limiting dilutions of human C1 were then added to aliquots of the cells and lysis was developed according to procedures described in Materials and Methods. Lower graph: $EAC\overline{14}$ were prepared with various limiting dilutions of C1. Aliquots of cells were then treated with LTAcx (250 µg/ml) or with buffer. After extensive washing, lysis was developed according to procedures described in Materials and Methods. Symbols: (●) Buffer treated cells; (○) LTAcx treated cells.


1.2
1.0
0.8
0.6
0.4
0.2
32,000
16,000
8,000
1.2
1.0
0.8
0.6
0.4
0.2
8,000
4,000
2,000
AVERAGE NUMBER OF SAC14/CELL
RECIPROCAL OF HUMAN C1 DILUTION

In addition to the above mentioned experiments, several other assays to elucidate the mechanism of inhibition were attempted. Unfortunately none of these experiments led to results that were consistent with any models attempting to explain how some complement cellular intermediates became refractory to complement lysis when pretreated with LTA. These experiments and their summarized data are presented below:

$\overline{C1}$ uptake by EA_{LTA}. EA_{LTA} were prepared with LTApcx at a concentration of 100 µg/ml using procedures described in the Materials and Methods. Buffer treated EA were also prepared at the same time. Human $\overline{C1}$ (approximately 1.0×10^{10} SFU/ml) was reacted with aliquots from each cell preparation and incubated for 15 minutes at 30°C. The cells were pelleted by centrifugation and the supernates analyzed for residual $\overline{C1}$ activity. Approximately 6.5×10^{9} SFU $\overline{C1}$/ml remained in the supernate of the buffer treated controls whereas approximately 6.8×10^{9} SFU $\overline{C1}$/ml were titrated in the supernate of the EA_{LTA} treated cells. Because values fluctuated by 5-8 % from one experiment to the next, this slight degree of enhancement was not considered significant.

Residual $C4^{HU}$ titration after preincubation of $C4^{HU}$ with $EA\overline{C1}_{LTA}$.

Human C4 (approximately 4.0×10^{9} SFU/ml) was added in equal volumes to $EA\overline{C1}$ which had been preincubated with either LTApcx (100 µg/ml) or with buffer. The mixture was incubated at 30° for 15 minutes and residual C4 activity was titrated as described in the Materials and Methods. EA were incubated with the C4 reagent as a negative control. Results indicated that there was approximately a 30% decrease in residual C4

titer of the supernates previously incubated with $EAC\overline{1}$ versus the negative control which consisted of C4 incubated with EA. However, both $EAC\overline{1}$ and $EAC\overline{1}_{LTA}$ consumed identical amounts of C4 (residual supernate C4 activity was 2.71×10^9 SFU/ml and 2.79×10^9 SFU/ml respectively). Therefore, it was concluded that LTA had no apparent effect on C4 uptake by $EAC\overline{1}$.

Residual $C2^{GP}$ titration after preincubation with $EAC\overline{14}$. Guinea pig C2 (approximately 1.5×10^{10} SFU/ml) was added in equal volumes to $EAC\overline{14}$ which had been preincubated with either LTApcx (100 µg/ml) or with buffer. The mixture was incubated at 30° for 12 minutes and residual C2 activity was titrated as described in Materials and Methods. EA were incubated with the C2 as a negative control. Results indicated that approximately 35% (5.3×10^9 SFU C2/ml) of the available C2 was utilized by the $EAC\overline{14}$ complexes and approximately 29% (4.4×10^9 SFU C2/ml) were utilized by the $EAC\overline{14}_{LTA}$ complexes. Despite the fact that the supernate from the C2 incubated with $EAC\overline{14}_{LTA}$ had slightly more residual C2 activity (approximately 71% of the C2 activity still remained in the supernate after incubation with $EAC\overline{14}_{LTA}$), a difference of only 6% is within experimental variance of this assay. Therefore, it was concluded that LTA had no apparent effect on C2 uptake by $EAC\overline{14}$.

Inhibition of lysis of EA by LTA from other bacterial sources. Additional evidence indicating that LTA might be primarily responsible for the C inhibition phenomenon came from hemolytic assays utilizing LTA from other sources. Dr. R. Doyle (Dept. of Microbiology and

Immunology, Univ. of Louisville) provided samples of LTA purified from Bacillus subtilis strain gta B290. Purified LTA from Lactobacillus casei ATCC 7469 was obtained from the Institute of Dental Research, Sydney, Australia, and Dr. A. S. Bleiweis (Dept. of Microbiology and Cell Science, Univ. of Florida) provided a sample of partially purified LTA from Streptococcus mutans strain AHT. Each preparation was mixed with EA; the cells were thoroughly washed and analyzed using the previously described techniques of PHA and susceptibility to whole complement lysis. As depicted in Table 11, all preparations contained material that reacted with anti-LTA by PHA and all such cells--especially those prepared with the purified L. casei--were more resistant to the hemolytic action of complement than were untreated controls.

TABLE 11

Percent Inhibition and PHA Titer of EAs
Treated with LTA Containing Extracts from Several Sources

Source of LTA[a]	Percent Inhibition[b]	PHA[c]
S. mutans BHT	40	1600
S. mutans AHT	35	1600
L. casei (ATCC 7469)	75	3200
B. subtilis (gta B290)	70	1600

[a]The LTA extracts from all sources were used at a concentration of 50 µg/ml in VBS.

[b]EAs were treated with the appropriate LTA-extract and hemolysis was developed by incubation of the cells with several dilutions of human C (37°/60 minutes). Values represent inhibition of CH_{50} units.

[c]PHA titers are expressed as the reciprocal of the final dilution of specific anti-LTA which caused hemagglutination.

DISCUSSION

Evidence has been provided for the inhibition of complement mediated lysis of target cells by an extracellular material obtained from Streptococcus mutans BHT. This material has been identified as lipoteichoic acid (LTA) and is a plasma membrane constituent of most gram positive bacteria (107,108).[1] Various gram positive bacteria isolated from the oral cavity differ in the amount of LTA they excrete into the external environment. S. mutans BHT is an example of a cariogenic streptococcus that not only produces copious amounts of LTA (1,20), but its ubiquitous nature provides for a constant inundation of LTA and other metabolites into the gingival crevices of the oral cavity. The presence of a complement reactive component in the microenvironment of the gingival crevices could result in any number of biological effects. Direct activation of the complement system (either classical or alternative) may result in the destruction of nearby "innocent bystander" cells. This is particularly true if the activator is cytophilic and thus capable of "sensitizing" nearby host cells. Activation of complement in the gingival crevices can also result in osteoclast-mediated bone resorbtion (14). This phenomenon is further complicated by the fact LTA and LPS (and ostensibly hybrid micells of the two) are

1 Some bacteria are known to lack LTA in their membranes but in these cases "LTA-like" molecules are inserted instead. Examples are the lipomannan of Micrococcus lysodeikticus (150) and the F-antigen of Diplococcus pneumoniae (115).

also capable of stimulating osteoclast mediated bone resorbtion (17). Even without profound activation of complement, the possession and release of complement inhibitory substances might confer a certain degree of survival value on the organisms producing them. Thus in the face of immunological challenge, the complement system may be blocked from reacting against the bacteria producing such factors. It may be more than coincidence that gram positive organisms such as Micrococcus lysodeikticus lacking LTA in their cell membranes are also susceptable to lysis by the synergism of lysozyme and complement (151). All other gram positives containing intact LTA in their membranes are notoriously resistant to complement lysis even in the presence of lysozyme (151).

Three lines of evidence have been obtained which suggest that the active inhibitory factor is lipteichoic acid (LTA). The inhibitor co-purified with LTA when extracellular material from spent culture was fractionated by gel-filtration and was purified by adsorbtion to phospholipid vesicles. Sheep erythrocytes which had been treated with S. mutans BHT extracellular extract became resistant to lysis by complement and they also became coated with LTA as judged by PHA using antibodies monospecific for purified LTA. The amount of LTA present on the cells paralleled the degree of lytic resistance that was acquired by the treatment. Purified LTA and LTA-rich fractions from other bacteria also caused sheep erythrocytes to become resistant to complement mediated hemolysis. Again, PHA assays indicated that cells which became resistant to lysis had LTA on their surfaces.

Experiments using crude extracellular LTA (LTAex) provided evidence for the consumption of whole human complement activity. When

preincubated with various concentrations of LTA, whole human sera lost complement activity in a dose-dependent fashion. Individual component titrations revealed that not only C3, but the early components C1, C4, and C2 were consumed to some degree. However, no C3 consumption was observed if the preincubation was performed with isolated C3 or in the presence of EDTA. If EGTA-Mg were substituted as the chelating agent, only a minimal restoration of C3 consuming activity was observed. These results indicated that not only were calcium and magnesium ions necessary for the anti-complementary activity, but there was a requirement for some factor(s) in whole sera as well. This "factor" is most likely natural antibody directed at LTA or some component of the crude extract. This resulted in the formation of a typical antigen-antibody complex with subsequent classical complement consumption.

Experiments using sheep erythrocytes in various stages of complement component fixation provided evidence that LTA was not only capable of spontaneously adsorbing to the surface of these cells, but also rendered many of the intermediates refractory to lysis. When sheep red blood cells, EA, or $EAC\overline{1}$ were treated with LTA, all became resistant to complement lysis. Lipoteichoic acid treated $EAC\overline{14}$ were somewhat less resistant to lysis and all cellular complement intermediates beyond $EAC\overline{14}$ were no longer protected.

Conversion of cells to hemolytic resistance by treatment with LTAcx can aid in the interpretation of the C2 consumption data depicted in Figure 4. As indicated, the degree of C2 consumption was disproportionate compared to loss of C1 and C4 activity. However, the commercially available human C2 used in these studies had a fairly low titer. As a result, the dilutions made after the preincubation step were not sufficient to prevent substantial amounts of the LTA from binding to

the cells and being expressed in the C2 titration. What appeared to be consumption of C2 activity was actually the inability of the complement system to lyse resistant cells. Because of the greater extent of dilution, the same phenomenon did not influence $\overline{C1}$, C4 and C3 titrations.

Because the $EAC\overline{142}$ and $EAC\overline{1423}\overline{567}$ intermediates were not effected by LTA, some component no longer necessary for their stability was a likely site of inhibition. C4 was probably not the site of attack since this component is a necessary part of the C3 convertase (152), and $EAC\overline{142}$ were not inhibited. Only $\overline{C1}$ is expendable after the $EAC\overline{142}$ complex is formed and thus C1 seemed to be the most likely candidate for the site of inhibition.

The first consideration was the possibility that LTA was causing inhibition of complement mediated lysis by blocking fixation of $\overline{C1}$ to antibodies specific for sheep erythrocytes or by blocking the site of antibody attachment. The fact that the inhibitor functioned equally well when it was presented either before or after the addition of specific antibodies to the cells indicated that blockage of antigenic sites was not the mechanism of inhibition. This experiment did not rule out the possibility that the inhibitory substance could react with the $\overline{C1}$ fixation sites on immunoglobulin molecules. However, Figure 10 shows that preincubation of LTAcx with anti-sheep E hemolysins did not decrease the hemolytic antibody titer of the serum. If LTA were capable of binding or inactivating immunoglobulin molecules (either specifically or non-specifically) then the titer of the antiserum should have been reduced as a result of treatment with the bacterial extract.

There was some speculation that LTA might inhibit complement mediated lysis by inducing some alteration in the structure of the target

cell membrane. However, one would expect all of the complement component intermediate cellular complexes to be equally affected by LTA, when in actuality this was not the case. It is possible that some of the complement components could block the attachment of the inhibitor to cell membranes so that the material would have no opportunity to cause membrane alteration. This is an unlikely possibility because even EAC$\overline{1423567}$ had LTA on their surfaces.

The highly purified LTA necessary for the final site of action and mechanism studies proved to be considerably more difficult to obtain than previously anticipated. As suggested by the results in Figures 13 and 15, and Tables 4 and 7, the Octyl Sepharose method of LTA purification did not sufficiently resolve the LTA from tenacious polysaccharide contamination. This method yielded almost quantitative recovery of LTA (as determined by PHAg) and also a significant portion of the total mass which was applied to the column. However, considering the contaminated nature of the final product even when an elution gradient was utilized, it was determined that a significant percentage of the mass was probably polysaccharide. Indeed, gas liquid chromatography of extracellular LTA purified by Octyl Sepharose (LTAosx) indicated that as much as 30% of the final weight was carbohydrate, presumably existing as polysaccharide (Figure 15, Table 7).

In contrast, the somewhat more elaborate method of purifying LTA by adsorbtion to phosphatidyl choline vesicles (PCV) yielded a product that was virtually devoid of all nucleic acid, protein, and carbohydrate contamination (Figure 15, Tables 5,6, and 7). Table 5 indicates that although approximately 7% of the radioactive ^{14}C used to label the PCV was lost during washing procedures (and ostensibly, a percentage

of bound LTA as well), over 92% of the label could be accounted for in the chloroform/methanol filtrate and the first filter washing. Only 0.004% of the label was present in the final product therefore eliminating phosphatidyl choline as a source of contamination. Figure 15 and Table 7 indicate that less than 5% polysaccharide contamination can be detected in the final product by gas liquid chromatography. Considering the unusual profiles obtained from the gas liquid chromatography of both cardiolipin and deacylated cardiolipin (Figure 15), it is likely that the percentage of contaminating polysaccharide in the final LTApex preparation is even less than 5%. As can be seen in Table 6, approximately 85% of the LTA in the original partially purified extract can be accounted for in the final product and washings. However, it should be cautioned that the method used for these determinations (PHAg) is semiquantitative at best and is only considered accurate to within one twofold dilution.

Although the percent protein of all partially purified samples was determined by amino acid analysis, unfortunately the tremendous quantity of purified material required in analysis for < 5% sensitivity in analysis, exceeded the total amount of purified material available. In fact after allocating fixed quantities of purified product for the various other quantitative and complement assays, the required 5-6 mg of purified LTA needed for amino acid analysis far exceeded the potential amount available from the LTAppx. For this reason, the Bio-Rad Protein Assay was used to estimate the total amount protein in each sample. As can be seen in Table 7, there was a relatively close correlation between values determined by amino acid analysis and those determined using the Bio-Rad Assay. It is therefore reasonable to

assume that the values given for the final products are at least a close indication of the total percent protein available in each product. Although the values may seem high, it should be remembered that (1) the total amount of protein available in the sample represents a lower limit for the accuracy of the assay, and (2) the standard protein curve (human albumin) used to convert optical density readings to µg of protein may not accurately correlate the reactions of the limited number of amino acid residues available in the final product. Attempts to verify these values with the biuret reaction (153) and the Lowry Protein Assay (154) were unsuccessful. Biuret was too insensitive whereas the Lowry proved to be unreliable due to its reaction with glycerol to give a false positive reaction. Despite this shortcoming, all other factors indicate that LTApcx represents the most highly purified LTA from S. mutans BHT that any laboratory has yet achieved.

The results from the final experiments to determine the site and mechanism of complement inhibition by LTA were equivocal. Like the purification of LTA, establishing the site and mechanism of inhibition proved to be considerably more challenging than anticipated. Preliminary data utilizing LTAcx quite consistently suggested that C1 was the site of action and interference with binding affinity (Clq dysfunction) or with esterase activity (C1s dysfunction) was the mechanism. These conclusions were based on the fact that $EAC\bar{1}\bar{4}_{LTA}$ but not $EAC\bar{1}\bar{4}\bar{2}_{LTA}$ were inhibited and also that the titer of fluid phase C1 preincubated with LTAcx was significantly reduced. Considering the polyanionic nature of LTA conferred by the polar polyglycerol phosphate backbone, it appeared that LTA represented a model system for polyanionic interference of C1 function. Such activity has been ascribed to dextran sulfate polyvinyl

sulfate, heparin, polyiosinic acid, chondroitin and many other polyanionic compounds (39,40) in addition to DNA, RNA (155,156), and carrageenin (157). It was dismaying to find that although LTApcx maintained anti-complementary activity with the appropriate cellular intermediates, all fluid phase inhibition of $\overline{Cl}$ was abrogated (Figures 17 and 18). All subsequent experiments attempting to define Clq, Cls, or $\overline{Cls}$ dysfunction were negative. The only experiments that gave suggestive results were the $\overline{Cl}$ transfer assays. Even here, instead of the anticipated inhibition of Cl transfer, over 20% enhancement of transfer was observed (Table 10). Thus, in light of these data obtained with purified LTA it was necessary to devise new molecular models to explain the mechanism of lytic inhibition of certain complement intermediates by highly purified LTA. Some possibilities are discussed below:

1). Attachment of LTA sterically blocks the affixation of $\overline{Cl}$ to the Fc portion of the immunoglobulin. Thus, if $\overline{Cl}$ does not attach properly, or is prevented from attaching at all, the complement cascade will never be initiated.

2). Although $\overline{Cls}$ activity was not effected fluid phase, perhaps such activity would be abrogated once the $\overline{Cl}$ molecule became associated with the cell membrane. If so, $EAC\overline{1}$ would no longer be capable of hydrolizing C4 or C2 again, the cascade would be terminated.

3). LTA directly interacts with fluid phase C4 or C2 thus preventing them from combining with the appropriate sites on the membrane.

4). The attachment of LTA leads to increased fluidity of the membrane resulting in the displacement of loosely attached molecules. If some of these less tenacious molecules include any of the early complement components, the physical loss of these components would terminate the lytic attack sequence.

5). The attachment of LTA to the cell membrane prevents the subsequent attachment of the C4 or C2 active fragments (i.e. C4b or C2a respectively). Thus, the activities of all complement components would remain intact and no observable dysfunction should be observed. However, if C4b or C2a were in the least impeded in their attachment to the cell membrane, these active fragments would rapidly decay and lose their ability to do so.

If the first model accurately portrayed the mechanism of inhibition, one would predict a decrease in $\overline{Cl}$ uptake by EA_{LTA}. This prediction was not corroborated by experimental results. In addition, this model would not explain the high degree of inhibition of cells in the $EA\overline{Cl}$ state where $\overline{Cl}$ is already attached.

If the second model were true, one would predict a decreased consumption of fluid phase C4 or C2 after preincubation with $EA\overline{Cl}_{LTA}$. Again, such was not the case. Neither residual C4 activity when incubated with $EA\overline{Cl}_{LTA}$ nor residual C2 activity when incubated with $EA\overline{Cl4}LTA$ was appreciably different from their buffer treated controls.

Model three would predict a decrease in fluid phase activity of C4 or C2 when preincubated with LTA. As demonstrated in Table 8, no such decrease in activity was observed.

Model four maintains that the attachment of LTA would somehow alter the membrane such that loosely attached components would be released more readily. The first problem with this model is that the attachment of the early complement components to the membrane is quite tenacious. In fact, some evidence suggests that membrane attachment of cytophillis C4b is accompanied by the formation of covalent bonds (158,159). Once attached, it seems unlikely that C4b would be readily

released. $C\bar{1}$ is not attached to the membrane at all, but rather is combined with the Fc region of the hemolysin antibody. Therefore, this model would predict that either $C\bar{1}$ is released from the antigen-antibody complex (very much akin to the predictions and shortcomings of model one) or that the entire antibody-C1 complex is released from the cell membrane (with or without the accompanying antigen). Such a mechanism is somewhat exotic, but not totally improbable. Recent evidence suggests that the binding of serum albumin, immunoglobulins, or complement can effect a release of phospholipids from liposomes (160, 161,162). Perhaps the attachment of LTA can likewise evoke such a release of cell membrane constituents and in the process, release the immune complexes as well. Experiments utilizing I^{131} labelled hemolysin antibody would demonstrate whether the antibody was maintained on the cell surface or released into the medium. Likewise, I^{131} labelled $C\bar{1}$ could be used to determine if $C\bar{1}$ were released.

Of all the proposed models, number five most likely portrays the actual mechanism of inhibition. This model asserts that the affixation of LTA to the surface of the cell delays or prevents the rapid association of C4b (or C2a) with its respective site on the cell membrane. As previously discussed, once C4 is cleaved by $C\bar{1}$, the cleavage results in the formation of a short-lived binding site on the C4b fragment. A high density of LTA on the surface of the cell might sequester C4b finding sites or perhaps change the electrostatic charge of the cell surface sufficiently to effect the kinetics of the C4b attachment. The end result in either case would be the nonproductive consumption of C4 molecules. This is consistant with the results from the residual C4 titration studies in which no alteration of C4 consumption was observed

when C4 was incubated with $EAC\overline{1}_{LTA}$. This model is also consistant with the fact no dysfunction of C1, C1q, C1s, $C\overline{1s}$, C4, or C2 could be demonstrated when incubated fluid phase with LTApcx.

This model would also predict that once C4b were attached to the membrane, subsequent addition of LTA should have significantly less impact on cascade disruption. As shown in Figure 17, this prediction coincides well with the facts. Percent inhibition of lysis drops from more than 65% in the case of EA treated with LTApcx (100 µg/ml) to less than 20% in the case of $EAC\overline{14}$ treated with the same concentration of LTApcx. Furthermore, $EAC\overline{142}_{LTA}$ are no longer inhibited as one would expect if the C4b and C2a binding sites were already secured.

Although all data thus far presented are consistant with this model, final proof would necessitate the I^{131} labelling of purified C4 and C2. Once labelled, one could determine if an excess of decayed C4b and C2a fragments were released into the media after preincubation with $EAC\overline{1}_{LTA}$ or $EAC\overline{14}_{LTA}$ respectively.

It is hoped that future research in this area may prove enlightening not only in expanding upon the mechanism of inhibition but also upon the specific role this extracellular metabolite plays in the inflammatory response of periodontal lesions.

It is apparent that the anti-complementary activity of LTA is not restricted to a single species or geneus (Table II) and it may very well be that LTA plays a significant role in protecting gram positive organisms from immunologic destruction. If so, LTA could be considered a type of "virulence" factor and those organisms that produce copious amounts of extracellular LTA (such as S. mutans BHT) would not only contribute to their own protection, but also to the protection of the

myriad of microorganisms in their immediate environment. Obviously, more research in this area is needed before such speculation can be substantiated with fact.

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BIOGRAPHICAL SKETCH

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Loui attended a parochial grade school (St. Thomas of Aquinas), a Jesuit preparatory high school (Scranton Preparatory School) and a college heavily influenced by Augustinian Catholicism (Villanova University).

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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